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History of Modern Biotechnology I

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VISIT…

Preface

The aim of the Advances of Biochemical Engineering/Biotechnology is to keep the reader informed on the recent progress in the industrial application of biology. Genetical engineering, metabolism ond bioprocess development including analytics, automation and new software are the dominant fields of interest. Thereby progress made in microbiology, plant and animal cell culture has been reviewed for the last decade or so.

The Special Issue on the History of Biotechnology (splitted into Vol. 69 and 70) is an exception to the otherwise forward oriented editorial policy. It covers a time span of approximately fifty years and describes the changes from a time with rather characteristic features of empirical strategies to highly developed and specialized enterprises. Success of the present biotechnology still depends on substantial investment in R&D undertaken by private and public investors, researchers, and enterpreneurs. Also a number of new scientific and business oriented organisations aim at the promotion of science and technology and the transfer to active enterprises, capital raising, improvement of education and fostering international relationships. Most of these activities related to modern biotechnology did not exist immediately after the war. Scientists worked in small groups and an established science policy didn't exist.

This situation explains the long period of time from the detection of the antibiotic effect by Alexander Fleming in 1928 to the rat and mouse testing by Brian Chain and Howart Florey (1940). The following developments up to the production level were a real breakthrough not only biologically (penicillin was the first antibiotic) but also technically (first scaled-up microbial mass culture under sterile conditions). The antibiotic industry provided the processing strategies for strain improvement (selection of mutants) and the search for new strains (screening) as well as the technologies for the aseptic mass culture and downstream processing. The process can therefore be considered as one of the major developments of that time what gradually evolved into "Biotechnology" in the late 1960s. Reasons for the new name were the potential application of a "new" (molecular) biology with its "new" (molecular) genetics, the invention of electronic computing and information science. A fascinating time for all who were interested in modern Biotechnology.

True gene technology succeeded after the first gene transfer into *Escherichia coli* in 1973. About one decade of hard work and massive investments were necessary for reaching the market place with the first recombinant product. Since then gene transfer in microbes, animal and plant cells has become a well-

established biological technology. The number of registered drugs for example may exceed some fifty by the year 2000.

During the last 25 years, several fundamental methods have been developed. Gene transfer in higher plants or vertebrates and sequencing of genes and entire genomes and even cloning of animals has become possible.

Some 15 microbes, including bakers yeast have been genetically identified. Even very large genomes with billions of sequences such as the human genome are being investigated. Thereby new methods of highest efficiency for sequencing, data processing, gene identification and interaction are available representing the basis of genomics – together with proteomics, a new field of biotechnology.

However, the fast developments of genomics in particular did not have just positive effects in society. Anger and fear began. A dwindling acceptance of "Biotechnology" in medicine, agriculture, food and pharma production has become a political matter. New legislation has asked for restrictions in genome modifications of vertebrates, higher plants, production of genetically modified food, patenting of transgenic animals or sequenced parts of genomes. Also research has become hampered by strict rules on selection of programs, organisms, methods, technologies and on biosafety indoors and outdoors.

As a consequence process development and production processes are of a high standard which is maintained by extended computer applications for process control and production management. GMP procedures are now standard and prerequisites for the registation of pharmaceuticals. Biotechnology is a safe technology with a sound biological basis, a high-tech standard, and steadily improving efficiency. The ethical and social problems arising in agriculture and medicine are still controversial.

The authors of the Special Issue are scientists from the early days who are familiar with the fascinating history of modern biotechnology. They have successfully contributed to the development of their particular area of specialization and have laid down the sound basis of a fast expanding knowledge. They were confronted with the new constellation of combining biology with engineering. These fields emerged from different backgrounds and had to adapt to new methods and styles of collaboration.

The historical aspects of the fundamental problems of biology and engineering depict a fascinating story of stimulation, going astray, success, delay and satisfaction.

I would like to acknowledge the proposal of the managing editor and the publisher for planning this kind of publication. It is his hope that the material presented may stimulate the new generations of scientists into continuing the rewarding promises of biotechnology after the beginning of the new millenium.

Zürich, August 2000 Armin Fiechter

The Natural Functions of Secondary Metabolites

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Secondary metabolites, including antibiotics, are produced in nature and serve survival functions for the organisms producing them. The antibiotics are a heterogeneous group, the functions of some being related to and others being unrelated to their antimicrobial activities. Secondary metabolites serve: (i) as competitive weapons used against other bacteria, fungi, amoebae, plants, insects, and large animals; (ii) as metal transporting agents; (iii) as agents of symbiosis between microbes and plants, nematodes, insects, and higher animals; (iv) as sexual hormones; and (v) as differentiation effectors. Although antibiotics are not obligatory for sporulation, some secondary metabolites (including antibiotics) stimulate spore formation and inhibit or stimulate germination. Formation of secondary metabolites and spores are regulated by similar factors. This similarity could insure secondary metabolite production during sporulation. Thus the secondary metabolite can: (i) slow down germination of spores until a less competitive environment and more favorable conditions for growth exist; (ii) protect the dormant or initiated spore from consumption by amoebae; or (iii) cleanse the immediate environment of competing microorganisms during germination.

Keywords. Secondary metabolite functions, Antibiosis, Differentiation, Metal transport, Sex hormones

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1 History of Secondary Metabolism

The practice of industrial microbiology (and biotechnology) has its roots deep in antiquity [1]. Long before their discovery, microorganisms were exploited to serve the needs and desires of humans, i. e., to preserve milk, fruit, and vegetables, and to enhance the quality of life with the resultant beverages, cheeses, bread, pickled foods, and vinegar. In Sumeria and Babylonia, the oldest biotechnology know-how, the conversion of sugar to alcohol by yeasts, was used to make beer. By 4000 BC, the Egyptians had discovered that carbon dioxide generated by the action of brewer's yeast could leaven bread, and by 100 BC, ancient Rome had over 250 bakeries which were making leavened bread. Reference to wine, another ancient product of fermentation, can be found in the Book of Genesis, where it is noted that Noah consumed a bit too much of the beverage. Wine was made in Assyria in 3500 BC As a method of preservation, milk was converted to lactic acid to make yoghurt, and also into kefir and koumiss using *Kluyveromyces* species in Asia. Ancient peoples made cheese with molds and bacteria. The use of molds to saccharify rice in the Koji process dates back at least to 700 AD By the 14th century AD, the distillation of alcoholic spirits from fermented grain, a practice thought to have originated in China or The Middle East, was common in many parts of the world. Interest in the mechanisms of these processes resulted in the later investigations by Louis Pasteur which not only advanced microbiology as a distinct discipline but also led to the development of vaccines and concepts of hygiene which revolutionized the practice of medicine.

In the seventeenth century, the pioneering Dutch microscopist Antonie van Leeuwenhoek, turning his simple lens to the examination of water, decaying matter, and scrapings from his teeth, reported the presence of tiny "animalcules", i. e., moving organisms less than one thousandth the size of a grain of sand. Most scientists thought that such organisms arose spontaneously from nonliving matter. Although the theory of spontaneous generation, which had been postulated by Aristotle among others, was by then discredited with respect to higher forms of life, it did seem to explain how a clear broth became cloudy via growth of large numbers of such "spontaneously generated microorganisms" as the broth aged. However, three independent investigators, Charles Cagniard de la Tour of France, Theodor Schwann, and Friedrich Traugott Kützing of Germany, proposed that the products of fermentation, chiefly ethanol and carbon dioxide, were created by a microscopic form of life. This concept was bitterly opposed by the leading chemists of the period (such as Jöns Jakob Berzelius, Justus von Liebig, and Friedrich Wöhler), who believed fermentation

was strictly a chemical reaction; they maintained that the yeast in the fermentation broth was lifeless, decaying matter. Organic chemistry was flourishing at the time, and these opponents of the living microbial origin were initially quite successful in putting forth their views. It was not until the middle of the nineteenth century that Pasteur of France and John Tyndall of Britain demolished the concept of spontaneous generation and proved that existing microbial life comes from preexisting life. It took almost two decades, from 1857 to 1876, to disprove the chemical hypothesis. Pasteur had been called on by the distillers of Lille to find out why the contents of their fermentation vats were turning sour. He noted through his microscope that the fermentation broth contained not only yeast cells but also bacteria that could produce lactic acid. One of his greatest contributions was to establish that each type of bioprocess is mediated by a specific microorganism. Furthermore, in a study undertaken to determine why French beer was inferior to German beer, he demonstrated the existence of strictly anaerobic life, i. e., life in the absence of air.

The field of biochemistry originated in the discovery by the Buchners that cell-free yeast extracts could convert sucrose into ethanol. Later, Chaim Weizmann of the UK applied the butyric acid bacteria, used for centuries for the retting of flax and hemp, for production of acetone and butanol. His use of *Clostridium* during World War I to produce acetone and butanol was the first nonfood bioproduct developed for large-scale production; with it came the problems of viral and microbial contamination that had to be solved. Although use of this process faded because it could not compete with chemical means for solvent production, it did provide a base of experience for the development of large scale cultivation of fungi for production of citric acid after the First World War, an aerobic process in which *Aspergillus niger* was used. Not too many years later, the discoveries of penicillin and streptomycin and their commercial development heralded the start of the antibiotic era.

For thousands of years, moldy cheese, meat, and bread were employed in folk medicine to heal wounds. It was not until the 1870s, however, that Tyndall, Pasteur, and William Roberts, a British physician, directly observed the antagonistic effects of one microorganism on another. Pasteur, with his characteristic foresight, suggested that the phenomenon might have some therapeutic potential. For the next 50 years, various microbial preparations were tried as medicines, but they were either too toxic or inactive in live animals. The golden era of antibiotics no doubt began with the discovery of penicillin by Alexander Fleming [2] in 1929 who noted that the mold *Penicillium notatum* killed his cultures of the bacterium *Staphylococcus aureus* when the mold accidentally contaminated the culture dishes. After growing the mold in a liquid medium and separating the fluid from the cells, he found that the cell-free liquid could inhibit the bacteria. He gave the active ingredient in the liquid the name "penicillin" but soon discontinued his work on the substance. The road to the development of penicillin as a successful drug was not an easy one. For a decade, it remained as a laboratory curiosity – an unstable curiosity at that. Attempts to isolate penicillin were made in the 1930s by a number of British chemists, but the instability of the substance frustrated their efforts. Eventually, a study began in 1939 at the Sir William Dunn School of Pathology of the University of Oxford by

Howard W. Florey, Ernst B. Chain, and their colleagues which led to the successful preparation of a stable form of penicillin and the demonstration of its remarkable antibacterial activity and lack of toxicity in mice. Production of penicillin by the strain of *Penicillium notatum* in use was so slow, however, that it took over a year to accumulate enough material for a clinical test on humans [3]. When the clinical tests were found to be successful, large-scale production became essential. Florey and his colleague Norman Heatley realized that conditions in wartime Britain were not conducive to the development of an industrial process for producing the antibiotic. They came to the US in the summer of 1941 to seek assistance and convinced the US Department of Agriculture in Peoria, Illinois, and several American pharmaceutical companies, to develop the production of penicillin. Heatley remained for a period at the USDA laboratories in Peoria to work with Moyer and Coghill.

Penicillin was originally produced in surface culture, but titers were very low. Submerged culture soon became the method of choice. The use of corn-steep liquor as an additive and lactose as carbon source stimulated production further. Production by a related mold, *Penicillium chrysogenum*, soon became a reality. Genetic selection began with *Penicillium chrysogenum* NRRL 1951, the well-known isolate from a moldy cantaloupe obtained in a Peoria market. It was indeed fortunate that the intense development of microbial genetics began in the 1940s when the microbial production of penicillin became an international necessity due to World War I. The early basic genetic studies concentrated heavily on the production of mutants and the study of their properties. The ease with which "permanent" characteristics of microorganisms could be changed by mutation and the simplicity of the mutation technique had tremendous appeal to microbiologists. Thus began the cooperative "strain-selection" program among workers at the U.S. Department of Agriculture in Peoria, the Carnegie Institution, Stanford University, and the University of Wisconsin, followed by the extensive individual programs that still exist today in industrial laboratories throughout the world. By the use of strain improvement and medium modifications, the yield of penicillin was increased 100-fold in 2 years. The penicillin improvement effort was the start of a long "engagement" between genetics and industrial microbiology which ultimately proved that mutation is the major factor involved in the hundred- to thousand-fold increases obtained in production of microbial metabolites.

Strain NRRL 1951 of *P. chrysogenum* was capable of producing 60 μg/ml of penicillin. Cultivation of spontaneous sector mutants and single-spore isolations led to higher-producing cultures. One of these, NRRL 1951 – 1325, produced 150 mg/ml. It was next subjected to X-ray treatment by Demerec of the Carnegie Institute at Cold Spring Harbor, New York, and mutant X-1612 was obtained, which formed 300 mg/ml. This tremendous cooperative effort among universities and industrial laboratories in England and the United States lasted throughout the war. Further clinical successes were demonstrated in both countries; finally in 1943 penicillin was used to treat those wounded in battle. Workers at the University of Wisconsin isolated ultraviolet-induced mutants of Demerec's strain. One of these, Wis. Q-176, which produced 550 mg/ml, is the parent of most of the strains used in industry today. The further development of

the "Wisconsin Family" of superior strains from Q-176 [4] led to strains producing over 1800 mg/ml. The new cultures isolated at the University of Wisconsin and in the pharmaceutical industry did not produce the yellow pigment which had been so troublesome in the early isolation of the antibiotic.

The importance of penicillin was that it was the first successful chemotherapeutic agent produced by a microbe. The tremendous success attained in the battle against disease with this compound not only led to the Nobel Prize being awarded to Fleming, Florey, and Chain, but to a new field of antibiotics research, and a new antibiotics industry. Penicillin opened the way for the development of many other antibiotics, and yet it still remains the most active and one of the least toxic of these compounds. Today, about 100 antibiotics are used to combat infections to humans, animals, and plants.

The advent of penicillin, which signaled the beginning of the antibiotics era, was closely followed by the discoveries of Selman A. Waksman, a soil microbiologist at Rutgers University. He and his students, especially H. Boyd Woodruff and Hubert Lechevalier, succeeded in discovering a number of new antibiotics from the the filamentous bacteria, the actinomycetes, such as actinomycin D, neomycin and the best-known of these new "wonder drugs", streptomycin. After its discovery in 1944, streptomycin's use was extended to the chemotherapy of many Gram-negative bacteria and to *Mycobacterium tuberculosis*. Its major impact on medicine was recognized by the award of the Nobel Prize to Waksman in 1952. As the first commercially successful antibiotic produced by an actinomycete, it led the way to the recognition of these organisms as the most prolific producers of antibiotics. Streptomycin also provided a valuable tool for studying cell function. After a period of time, during which it was thought to act by altering permeability, its interference with protein synthesis was recognized as its primary effect. Its interaction with ribosomes provided much information on their structure and function; it not only inhibits their action but also causes misreading of the genetic code and is required for the function of ribosomes in streptomycin-dependent mutants.

The development of penicillin fermentation in the 1940s marked the true process beginning of what might be called the golden age of industrial microbiology, resulting in a large number of microbial primary and secondary metabolites of commercial importance. Primary metabolism involves an interrelated series of enzyme-mediated catabolic, amphibolic, and anabolic reactions which provide biosynthetic intermediates and energy, and convert biosynthetic precursors into essential macromolecules such as DNA, RNA, proteins, lipids, and polysaccharides. It is finely balanced and intermediates are rarely accumulated. The most important primary metabolites in the bio-industry are amino acids, purine nucleotides, vitamins, and organic acids. Of all the traditional products made by bioprocess, the most important to human health are the secondary metabolites (idiolites). These are metabolites which: (i) are often produced in a developmental phase of batch culture (idiophase) subsequent to growth; (ii) have no function in growth; (iii) are produced by narrow taxonomic groups of organisms; (iv) have unusual and varied chemical structures; and (v) are often formed as mixtures of closely related members of a chemical family. Bu'Lock [5] interpreted secondary metabolism as a manifestation of differentiation which

accompanies unbalanced growth. In nature, their functions serve the survival of the strain, but when the producing microorganisms are grown in pure culture, the secondary metabolites have no such role. Thus, production ability in industry is easily lost by mutation ("strain degeneration"). In general, both the primary and the secondary metabolites of commercial interest have fairly low molecular weights, i. e., less than 1500 daltons. Whereas primary metabolism is basically the same for all living systems, secondary metabolism is mainly carried out by plants and microorganisms and is usually strain-specific. The best-known secondary metabolites are the antibiotics. More than 5000 antibiotics have already been discovered, and new ones are still being found at a rate of about 500 per year. Most are useless; they are either too toxic or inactive in living organisms to be used. For some unknown reason, the actinomycetes are amazingly prolific in the number of antibiotics they can produce. Roughly 75% of all antibiotics are obtained from these filamentous prokaryotes, and 75% of those are in turn made by a single genus, *Streptomyces*. Filamentous fungi are also very active in antibiotic production. Antibiotics have been used for purposes other than human and animal chemotherapy, such as the promotion of growth of farm animals and plants and the protection of plants against pathogenic microorganisms.

Cooperation on the development of the penicillin and streptomycin productions into industrial processes at Merck & Co., Princeton University, and Columbia University led to the birth of the field of biochemical engineering. Following on the heels of the antibiotic products was the development of efficient microbial processes for the manufacture of vitamins (riboflavin, cyanocobalamine, biotin), plant growth factors (gibberellins), enzymes (amylases, proteases, pectinases), amino acids (glutamate, lysine, threonine, phenylalanine, aspartic acid, tryptophan), flavor nucleotides (inosinate, guanylate), and polysaccharides (xanthan polymer), among others. In a few instances, processes have been devised in which primary metabolites such as glutamic acid and citric acid accumulate after growth in very large amounts. Cultural conditions are often critical for their accumulation and in this sense, their accumulation resembles that of secondary metabolites.

Despite the thousands of secondary metabolites made by microorganisms, they are synthesized from only a few key precursors in pathways that comprise a relatively small number of reactions and which branch off from primary metabolism at a limited number of points. Acetyl-CoA and propionyl-CoA are the most important precursors in secondary metabolism, leading to polyketides, terpenes, steroids, and metabolites derived from fatty acids. Other secondary metabolites are derived from intermediates of the shikimic acid pathway, the tricarboxylic acid cycle, and from amino acids. The regulation of the biosynthesis of secondary metabolites is similar to that of the primary processes, involving induction, feedback regulation, and catabolite repression [6].

There was a general lack of interest in the penicillins in the 1950s after the exciting progress made during World War II. By that time, it was realized that *P. chrysogenum* could use additional acyl compounds as side-chain precursors (other than phenylacetic acid for penicillin G) and produce new penicillins, but only one of these, penicillin V (phenoxymethylpenicillin), achieved any

commercial success. Its commercial application resulted from its stability to acid which permitted oral administration, an advantage it held over the accepted article of commerce, penicillin G (benzylpenicillin). Research in the penicillin field in the 1950s was mainly of an academic nature, probing into the mechanism of biosynthesis. During this period, the staphylococcal population was building up resistance to penicillin via selection of penicillinase-producing strains and new drugs were clearly needed to combat these resistant forms. Fortunately, two developments occurred which led to a rebirth of interest in the penicillins and related antibiotics. One was the discovery by Koichi Kato [7] of Japan in 1953 of the accumulation of the "penicillin nucleus" in *P. chrysogenum* broths to which no side-chain precursor had been added. In 1959, Batchelor et al. [8] isolated the material (6-aminopenicillanic acid) which was used to make "semi-synthetic" (chemical modification of a natural product) penicillins with the beneficial properties of resistance to penicillinase and to acid, plus broad-spectrum antibacterial activity. The second development was the discovery of "synnematin B" in broths of *Cephalosporium salmosynnematum* by Gottshall et al. [9] in Michigan, and that of "cephalosporin N" from *Cephalosporium* sp. by Brotzu in Sardinia and its isolation by Crawford et al. [10] at Oxford. It was soon found that these two molecules were identical and represented a true penicillin possessing a side-chain of D-α-aminoadipic acid. Thus, the name of this antibiotic was changed to penicillin N. Later, it was shown that a second antibiotic, cephalosporin C, was produced by the same *Cephalosporium* strain producing penicillin N [11]. Abraham, Newton, and coworkers found the new compound to be related to penicillin N in that it consisted of a β-lactam ring attached to a side chain of D-α-aminoadipic acid. It differed, however, from the penicillins in containing a six-membered dihydrothiazine ring in place of the five-membered thiazolidine ring of the penicillins.

Although cephalosporin C contained the β-lactam structure, which is the site of penicillinase action, it was a poor substrate and was essentially not attacked by the enzyme, was less toxic to mice than penicillin G, and its mode of action was the same; i. e., inhibition of cell wall formation. Its disadvantage lied in its weak activity; it had only 0.1 % of the activity of penicillin G against sensitive staphylococci, although its activity against Gram-negative bacteria equaled that of penicillin G. However, by chemical removal of its D-α-aminoadipidic acid side chain and replacement with phenylacetic acid, a penicillinase-resistant semisynthetic compound was obtained which was 100 times as active as cephalosporin C. Many other new cephalosporins with wide antibacterial spectra were developed in the ensuing years, making the semisynthetic cephalosporins the most important group of antibiotics. The stability of the cephalosporins to penicillinase is evidently a function of the dihydrothiazine ring since: (i) the D-α-aminoadipic acid side chain does not render penicillin N immune to attack; and (ii) removal of the acetoxy group from cephalosporin C does not decrease its stability to penicillinase. Cephalosporin C competitively inhibits the action of penicillinase from *Bacillus cereus* on penicillin G. Although it does not have a similar effect on the *Staphylococcus aureus* enzyme, certain of its derivatives do. Cephalosporins can be given to some patients who are sensitive to penicillins.

The antibiotics form a heterogeneous assemblage of biologically active molecules with different structures [12, 13] and modes of action [14]. Since 1940, we have witnessed a virtual explosion of new and potent molecules which have been of great use in medicine, agriculture, and basic research. Over 50,000 tons of these metabolites are produced annually around the world. However, the search for new antibiotics continues in order to: (i) combat naturally resistant bacteria and fungi, as well as those previously susceptible microbes that have developed resistance; (ii) improve the pharmacological properties of antibiotics; (iii) combat tumors, viruses, and parasites; and (iv) discover safer, more potent, and broader spectrum antibiotics. All commercial antibiotics in the 1940s were natural, but today most are semisynthetic. Indeed, over 30,000 semisynthetic β-lactams (penicillins and cephalosporins) have been synthesized.

The selective action that microbial secondary metabolites exert on pathogenic bacteria and fungi was responsible for ushering in the antibiotic era, and for 50 years we have benefited from this remarkable property of these "wonder drugs." The success rate was so impressive that secondary metabolites were the predominant molecules used for antibacterial, antifungal, and antitumor chemotherapy. As a result, the pharmaceutical industry screened secondary metabolites almost exclusively for such activities. This narrow view temporarily limited the application of microbial metabolites in the late 1960s. Fortunately, the situation changed and industrial microbiology entered into a new era in the 1970 – 1980 period in which microbial metabolites were studied for diseases previously reserved for synthetic compounds, i. e., diseases that are not caused by other bacteria, fungi or tumors [15].

With great vision, in the 1960s Hamao Umezawa began his pioneering efforts to broaden the scope of industrial microbiology to low molecular weight secondary metabolites which had activities other than, or in addition to, antibacterial, antifungal, and antitumor action. He and his colleagues at the Institute of Microbial Chemistry in Tokyo focused on enzyme inhibitors [16] and over the years discovered, isolated, purified, and studied the in vitro and in vivo activity of many of these novel compounds. Similar efforts were conducted at the Kitasato Institute in Tokyo led by Satoshi Omura [17]. The anti-enzyme screens led to acarbose, a natural inhibitor of intestinal glucosidase, which is produced by an actinomycete of the genus *Actinoplanes* and which decreases hyperglycemia and triacylglycerol synthesis in adipose tissue, liver, and the intestinal wall of patients with diabetes, obesity, and type IV hyperlipidaemia. Even more important enzyme inhibitors which have been well accepted include those for medicine (clavulanic acid, lovastatin) and agriculture (polyoxins, phosphinothricins). Clavulanic acid is a penicillinase inhibitor which is used in combination with penicillinase-sensitive penicillins. Lovastatin (mevinolin) is a remarkably successful fungal product which acts as a cholesterol-lowering agent in animals. It is produced by *Aspergillus terreus* and, in its hydroxyacid form (mevinolinic acid), is a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase from liver.

Broad screening led to the development of ergot alkaloids for various medical uses (uterocontraction, migraine headaches, etc.), monensin as a coccidiostat, gibberellins as a plant growth stimulators, zearelanone as an estrogenic agents

in animals, phosphinothricins as herbicides, spinosyns as insecticides, and cyclosporin as an immunosuppressant. Cyclosporin A virtually revolutionized the practice of organ transplantation in medicine. Broad screening allowed the polyether monensin to take over the coccidiostat market from synthetic compounds and avermectin to do the same with respect to the antihelmintic market. Direct in vivo screening of reaction mixtures against nematodes in mice led to the major discovery of the potent activity of the avermectins against helminths causing disease in animals and humans. Avermectin's antihelmintic activity was an order of magnitude greater than previously developed synthetic compounds. The above successes came about in two ways: (i) broad screening of known compounds which had failed as useful antibiotics; and (ii) screening of unknown compounds in process media for enzyme inhibition, inhibition of a target pest, or other activities. Both strategies had one important concept in common, i. e., that microbial metabolites have activities other than, or in addition to, inhibition of other microbes. Today's screens are additionally searching for receptor antagonists and agonists, antiviral agents, anti-inflammatory drugs, hypotensive agents, cardiovascular drugs, lipoxygenase inhibitors, antiulcer agents, aldose reductase inhibitors, antidiabetes agents, and adenosine deaminase inhibitors, among others.

Recombinant DNA technology has been applied to the production of antibiotics. Many genes encoding individual enzymes of antibiotic biosynthesis have been cloned and expressed at high levels in heterologous microorganisms. Continued efforts in the application of recombinant DNA technology to bioengineering have led to overproduction of limiting enzymes of important biosynthetic pathways, thereby increasing production of the final products. In addition, a large number of antibiotic-resistance genes from antibiotic-producing organisms have been cloned and expressed. Some antibiotic biosynthetic pathways are encoded by plasmid-borne genes (e. g., methylenomycin A). Even when the antibiotic biosynthetic pathway genes of actinomycetes are chromosomal (the usual situation), they are clustered, which facilitates transfer of an entire pathway in a single manipulation. The genes of the actinorhodin pathway, normally clustered on the chromosome of *Streptomyces coelicolor,* were transferred en masse on a plasmid to *Streptomyces parvulus* and were expressed in the latter organism. Even in fungi, pathway genes are sometimes clustered, such as the penicillin genes in *Penicillium* or the aflatoxin genes in *Aspergillus*. For the discovery of new or modified products, recombinant DNA techniques have been used to introduce genes coding for antibiotic synthetases into producers of other antibiotics or into nonproducing strains to obtain modified or hybrid antibiotics. Gene transfer from a streptomycete strain producing the isochromanequinone antibiotic actinorhodin into strains producing granaticin, dihydrogranaticin, and mederomycin (which are also isochromanequinones) led to the discovery of two new antibiotic derivatives, mederrhodin A and dihydrogranatirhodin [18]. Since that development, many novel polyketide secondary metabolites have been obtained by cloning DNA fragments from one polyketide producer into various strains of other streptomycetes [19].

For many years, basic biologists were uninterested in secondary metabolism. There were so many exciting discoveries to be made in the area of primary

metabolism and its control that secondary metabolism was virtually ignored; study of this type of non-essential ("luxury") metabolism was left to industrial scientists and academic chemists and pharmacognocists. Today, the situation is different. The basic studies on *Escherichia coli* and other microorganisms elucidated virtually all of the primary metabolic pathways and most of the relevant regulatory mechanisms; many of the enzymes were purified, and the genes encoding them isolated, cloned, and sequenced. The frontier of expanding knowledge is now secondary metabolism which poses many questions of considerable interest to science: What are the functions of idolites in nature? How are the pathways controlled? What are the origins of secondary metabolism genes? How is it that the same genes, enzymes, and pathways exist in organisms as different as the eukaryote *Cephalosporium acremonium* and the prokaryote, *Flavobacterium* sp.? What are the origins of the resistance genes which producing organisms use to protect themselves from suicide? Are these the same genes as those found in clinically-resistant bacteria? The use of microorganisms and their antibiotics as tools of basic research is mainly responsible for the remarkable advances in the fields of molecular biology and molecular genetics. Fortunately, molecular biology has produced tools with which to answer these questions. It is clear that basic mechanisms controlling secondary metabolism are now of great interest to many academic (and industrial) laboratories throughout the world.

Natural products have been an overwhelming success in our society. It has been stated that the doubling of the human life span in the twentieth century is due mainly to the use of plant and microbial secondary metabolites [20]. They have reduced pain and suffering and revolutionized medicine by allowing the transplantation of organs. They are the most important anticancer agents. Over 60% of approved and pre-NDA (new drug applications) candidates are either natural products or related to them, even when not including biologicals such as vaccines and monoclonal antibodies [21]. Almost half of the best-selling pharmaceuticals are natural or related to natural products. Often, the natural molecule has not been used itself, but served as a lead molecule for manipulation by chemical or genetic means. Natural product research is at its highest level as a consequence of unmet medical needs, the remarkable diversity of natural compound structures and activities, their use as biochemical probes, the development of novel and sensitive assay methods, improvements in the isolation, purification, and characterization of natural products, and new production methods [22]. It is clear that, although the microbe has contributed greatly to the benefit of mankind, we have merely scratched the surface of the potential of microbial activity.

2
Secondary Metabolites Have Functions in Nature

It was once popular to think that secondary metabolites were merely laboratory artifacts but today there is no doubt that secondary metabolites are natural products. Over 40% of filamentous fungi and actinomycetes produce antibiotics when they are freshly isolated from nature. In a survey of 111 coprophilous fungal

species (representing 66 genera) colonizing dung of herbivorous vertebrates, over 30% were found to produce antifungal agents [23]. Foster et al. [24] reported that 77% of soil myxobacteria produced antibiotic activity against *Micrococcus luteus*. This confirms the earlier figure of 80% by Reichenbach et al. [25]. Many of these myxobacteria showed antifungal activity and a few were active against Gram-negative bacteria. In an extensive survey of gliding bacteria done between 1975 and 1991, it was found that bioactive metabolites were made by 55% of bacteriolytic myxobacteria, 95% of the cellulolytic myxobacteria (genus *Sorangium*), 21% of the *Cytophaga*-like bacteria, and 21% of *Lysobacter* [26].

Secondary metabolites are mainly made by filamentous microorganisms undergoing complex schemes of morphological differentiation, e. g., molds make 17% of all described antibiotics and actinomycetes make 74% [27]. Members of the unicellular bacterial genus *Bacillus* are also quite active in this respect. Some species are prolific in secondary metabolism: strains of *Streptomyces hygroscopicus* produce over 180 different secondary metabolites [28]. Estimates of the number of microbial secondary metabolites thus far discovered vary from 8000 up to 50,000 [12, 17, 26, 29–31]. Many secondary metabolites are made by plants. Unusual chemical structures of microbial and plant metabolites include β-lactam rings, cyclic peptides, and depsipeptides containing "unnatural" and non-protein amino acids, unusual sugars and nucleosides, unsaturated bonds of polyacetylenes and polyenes, covalently bound chlorine and bromine; nitro-, nitroso-, nitrilo-, and isonitrilo groups, hydroxamic acids, diazo compounds, phosphorus as cyclic triesters, phosphonic acids, phosphinic acids, and phosphoramides, 3-,4- and 7-membered rings, and large rings of macrolides, macrotetralides, and arisamycines. Their enormous diversity includes 22,000 terpenoids [32].

Soil, straw, and agricultural products often contain antibacterial and antifungal substances. These are usually considered to be "mycotoxins," but they are nevertheless antibiotics. Indeed, one of our major public health problems is the natural production of such toxic metabolites in the field and during storage of crops. The natural production of ergot alkaloids by the sclerotial (dormant overwintering) form of *Claviceps* on the seed heads of grasses and cereals has led to widespread and fatal poisoning ever since the Middle Ages [33]. Natural soil and wheat-straw contain patulin [34] and aflatoxin is known to be produced on corn, cottonseed, peanuts, and tree nuts in the field [35]. These toxins cause hepatotoxicity, teratogenicity, immunotoxicity, mutation, cancer, and death [36]. Corn grown in the tropics or semitropics always contains aflatoxin [37]. At least five mycotoxins of *Fusarium* have been found to occur naturally in corn: moniliformin, zearalenone, deoxynivalenol, fusarin C, and fumonisin [38]. Trichothecin is found in anise fruits, apples, pears, and wheat [39]. Sambutoxin produced by *Fusarium sambucinum* and *Fusarium oxysporum* was isolated from rotten potato tubers in Korea [40]. Microbially produced siderophores have been found in soil [41] and microcins (enterobacterial antibiotics) have been isolated from human fecal extracts [42]. The microcins are thought to be important in colonization of the human intestinal tract by *Escherichia coli* early in life. Cyanobacteria cause human and animal disease by producing cyclic heptapeptides (microcystins by *Microcystis*) and a cyclic pentapeptide (nodularin by *Nodularia*)

in water supplies [43]. Antibiotics are produced in unsterilized, unsupplemented soil, in unsterilized soil supplemented with clover and wheat straws, in mustard, pea, and maize seeds, and in unsterilized fruits [44]. A further indication of natural antibiotic production is the possession of antibiotic-resistance plasmids by most soil bacteria [45]. Nutrient limitation is the usual situation in nature resulting in very low bacterial growth rates, e. g., 20 days in deciduous woodland soil [46]. Low growth rates favor secondary metabolism.

The widespread nature of secondary metabolite production and the preservation of their multigenic biosynthetic pathways in nature indicate that secondary metabolites serve survival functions in organisms that produce them. There are a multiplicity of such functions, some dependent on antibiotic activity and others independent of such activity. Indeed in the latter case, the molecule may possess antibiotic activity but may be employed by a producing microorganism for an entirely different purpose. Some useful reviews on secondary metabolism have appeared in recent years [23, 47 – 49]. Examples of marine secondary metabolites playing a role in marine ecology have been given by Jensen and Fenical [50].

The view that secondary metabolites act by improving the survival of the producer in competition with other living species has been expressed more and more in recent years [51, 52]. Arguments are as follows:

1. Only organisms lacking an immune system are prolific producers of these compounds which act as an alternative defense mechanism.
2. The compounds have sophisticated structures, mechanisms of action, and complex and energetically expensive pathways [53].
3. Soil isolates produce natural products, most of which have physiological properties.
4. They are produced in nature and act in competition between microorganisms, plants and animals [44, 54].
5. Clustering of biosynthetic genes, which would only be selected for if the product conferred a selective advantage, and the absence of non-functional genes in these clusters.
6. The presence of resistance and regulatory genes in these clusters.
7. The clustering of resistance genes in non-producers.
8. The temporal relationship between antibiotic formation and sporulation [53, 55] due to sensitivity of cells during sporulation to competitors and the need for protection when a nutrient runs out.

Williams and coworkers call this "plieotropic switching," i. e., a way to express concurrently both components of a two-pronged defense strategy when survival is threatened. They contend that the secondary metabolites act via specific receptors in competing organisms. According to Gloer [23], fungal secondary metabolites function in plant disease, insect disease, poisoning of animals, resistance to infestation and infection by other microbes, and antagonism between species.

It has been proposed that antibiotics and other secondary metabolites, originally produced by chemical (non-enzymatic) reactions, played important evolutionary roles in effecting and modulating prehistoric reactions (e. g., primitive transcription and translation) by reacting with receptor sites in primi-

tive macromolecular templates made without enzymes [56]. Later on, the small molecules were thought to be replaced by polypeptides but retained their abilities to bind to receptor sites in nucleic acids and proteins. Thus, they changed from molecules with a function in synthesis of macromolecules to antagonists of such processes, e.g., as antibiotics, enzyme inhibitors, receptor antagonists, etc. As evidence, Davies [56] cites examples in which antibiotics are known to stimulate gene transfer, transposition, transcription, translation, cell growth, and mutagenesis.

3 Functions

3.1 Agents of Chemical Warfare in Nature

According to Cavalier-Smith [57], secondary metabolites are most useful to the organisms producing them as competitive weapons and the selective forces for their production have existed even before the first cell. The antibiotics are more important than macromolecular toxins such as colicins and animal venoms because of their diffusibility into cells and broader modes of action.

3.1.1 *Microbe vs Microbe*

One of the first pieces of evidence indicating that one microorganism produces an antibiotic against other microorganisms and that this provides for survival in nature was published by Bruehl et al. [58]. They found that *Cephalosporium gramineum*, the fungal cause of stripe disease in winter wheat, produces a broad spectrum antifungal antibiotic of unknown structure. Over a three year period, more than 800 isolates were obtained from diseased plants, each of which was capable of producing the antibiotic in culture. On the other hand, ability to produce the antibiotic was lost during storage on solid medium at 6 °C. Thus, antibiotic production was selected for in nature but was lost in the test tube, the selection being exerted during the saprophytic stage in soil. These workers further showed that antibiotic production in the straw-soil environment aided in the survival of the producing culture and markedly reduced competition by other fungi.

Antagonism between competing fungi in nature has been demonstrated in virtually every type of fungal ecosystem including coprophilous, carbinocolous, lignicolous, fungicolous, phylloplane, rhizosphere, marine, and aquatic [59]. Of 150 selected coprophilous fungal species representing 68 genera, 60 % displayed fungal inhibition involving diffusible products.

Gliocladium virens inhibits the growth of *Pythium ultimum*, a phytopathogen, in the soil by production of the antibiotic, gliovirin [45]. A nonproducing mutant was overgrown in culture by *P. ultimum* and did not protect cotton seedlings from damping off disease in soil infested with *P. ultimum*. A superior-producing mutant was more inhibitory than the parent culture and showed parental

efficiency in disease suppression even though its growth rate was lower than that of the parent. Cell walls of the phytopathogenic fungus, *Botryitis cinerea*, induce in *Trichoderma harzianum* the formation of chitinase, β-1,3-glucanase, and the membrane-channeling antibiotics, peptaibols (= trichorzianines). The antibiotics and enzymes act synergistically in inhibiting spore germination and hyphal extension in *B. cinerea* [60].

Another example involves the parasitism of one fungus on another. The parasitism of *Monocillium nordinii* on the pine stem rust fungi *Cronartium coleosporioides* and *Endocronartium harkenssii* is due to production of the antifungal antibiotics monorden and the monocillins [61].

Competition between bacteria is also effected via antibiotics. Agrocin 84, a plasmid-coded antibiotic of *Agrobacterium rhizogenes*, is an adenine derivative which attacks strains of plant pathogenic agrobacteria. It is used commercially in the prevention of crown gall and acts by killing the pathogenic forms [62].

An interesting relationship exists between myxobacteria and their bacterial "diet." Myxobacteria live on other bacteria, and to grow on these bacteria they require a high myxobacterial cell density. This population effect is primarily due to the need for a high concentration of lytic enzymes and antibiotics in the local environment. Thus, *Myxococcus xanthus* fails to grow on *E. coli* unless more than 10^7 myxobacteria/ml are present [63]. At these high cell concentrations, the parent grows but a mutant which cannot produce antibiotic TA fails to grow. This indicates that the antibiotic is involved in the killing and nutritional use of other bacteria. Between 60% and 80% of myxobacteria produce antibiotics [64]. In nature, different myxobacteria establish their own territory when they are about to form fruiting bodies [65]. The same phenomenon can be repeated in the laboratory when vegetative swarms of two types come together on a solid surface. Each type apparently recognizes the other type and establishes its own site by the use of antagonistic agents. When *Myxococcus xanthus* was mixed with *Myxococcus virescens*, the latter predominated over the former by producing an extracellular bacteriocin which kills *M. xanthus*. However, *M. xanthus* can inhibit the growth and development of *M. virescens* by excreting an inhibitory agent.

Antibiotic production was crucial in competition studies carried out in autoclaved sea water [66]. Four antibiotic-producing marine bacteria and three non-producing marine bacteria were grown in pairs or three-membered cultures. In every case of a non-producer and a producer pair, the non-producer disappeared. In five pairs of producer cultures, one producer survived and the other did not in four of the cases. When non-producers were paired or combined in three-membered cultures, all survived. In three-membered cultures including at least one producer, the producer always survived. This work supports the amensalism concept that antibiotic production aids in survival by killing or inhibiting other strains. When the bacteriocin-producing strain LPC010 of *Lactobacillus plantarum* was inoculated into a green olive bioprocesses, it produced its bacteriocin and dominated over the natural flora of lactic acid bacteria throughout the 12-week process [67]. On the other hand, its bacteriocin-negative mutant failed to persist for even 7 weeks.

Erwinia carotovora subsp. *betavasculorum* is a wound pathogen causing vascular necrosis and root rot of sugar beet. It produces a broad-spectrum anti-

biotic which is the principal determinant allowing it to compete successfully in the potato against the antibiotic-sensitive *E. carotovora* subsp. *carotovora* strains. Complete correlation was observed between antibiotic production in vitro and inhibition of subsp. *carotovora* strains in the plant [68].

Competition also occurs between strains of a single species. Phenazine production by *Pseudomonas phenazinium* results in smaller colonies and lower maximum cell densities (but not lower growth rates) than those of non-producing mutants [69]. Furthermore, the viability of non-producing mutants in various nutrient-limited media is higher than that of the producing parent. Despite these apparent deficiencies, the producing strain wins out in a mixed culture in the above media. The parental strain is able to use its phenazine antibiotic to kill the non-producing cells and, due to its resistance to the antibiotic, the parent survives.

3.1.2 *Bacteria vs Amoebae*

Since protozoa use bacteria as food [70] and utilize these prokaryotes to concentrate nutrients for them, it is not surprising that mechanisms have evolved to protect the bacteria against protozoans such as amoebae. Over 50 years ago, Singh [71] noted that antibiotically-active pigments from *Serratia marcescens* and *Chromobacterium violaceum* (prodigiosin and violacein, respectively) protect these species from being eaten by amoebae; in the presence of the pigment, the protozoa either encyst or die. Of interest is the fact that nonpigmented *S. marcescens* cells are consumed by amoebae but pigmented cells are not. These experiments have been extended to other bacteria such as *Pseudomonas pyocyanea* and *Pseudomonas aeruginosa* and to microbial products such as pyocyanine, penicillic acid, phenazines, and citrinin [72–74]. These findings show that antagonism between amoebae and bacteria in nature is crucially affected by the ability of the latter to produce antibiotics. Since bacteria appear to be a major source of nutrients for planktonic algae especially at low light intensities [75], we can anticipate the discovery of antibiotics being produced by bacteria against algae.

3.1.3 *Microorganisms vs Higher Plants*

More than 150 microbial compounds called phytotoxins or phytoaggressins that are active against plants have been reported and the structures of over 40 are known [76]. Many such compounds (e.g., phaseolotoxin, rhizobitoxine, syringomycin, syringotoxin, syringostatin, tropolone, and fireblight toxin) show typical antibiotic activity against other microorganisms and are thus both antibiotics and phytotoxins. These include many phytotoxins of *Pseudomonas* which are crucial in the pathogenicity of these strains against plants [77]. These toxins, which induce chlorosis in plant tissue [78], include tabtoxinine-β-lactam (a glutamine antagonist produced by *Pseudomonas syringae* pv. "tabaci" and *Pseudomonas coronofaciens* which causes wildfire in tobacco and halo blight

in oats, respectively) and phaseolotoxin, a tripeptide arginine antimetabolite of *P. syringae* pv. "*phaseolicola*" which causes halo blight in French beans. Phaseolotoxins not only induce chlorosis but are necessary for the systematic spread of *P. syringae* pv. "*phaseolicola*" throughout the plant [79]. Other phytotoxic antibiotics include syringomycin and the toxic peptides of *Pseudomonas glycinea* and *Pseudomonas tomato* [80]. Syringomycin, a cyclic lipodepsinonapeptide produced by the plant pathogenic *Pseudomonas syringae* pv. *syringae* is phytotoxic, is involved in bacterial canker of stone fruit trees and holcus spot of maize, and is also a broad-spectrum antibiotic against procaryotes and eucaryotes including *Geotrichum candidum* [81]. Proof of the role of antibiotics as plant toxins has been provided in the case of syringomycin [82] which disrupts ion transfer across the plasmalemma of plant cells. Syringomycin synthetases are encoded by a series of genes, including *syrB*, which appears to encode a subunit of one or both of two proteins, namely SR4 (350 kDa) and SR5 (130 kDa). Using a *syrB::lacZ* fusion, it was found that the gene is transcriptionally activated by plant metabolites with signal activity, e. g., arbutin, phenyl-β-D-glucopyranoside, salicin, aescalin, and helicin, which are all produced by plants susceptible to the pathogen. Activators of genes involved in virulence of *Agrobacterium tumefaciens* (acetosyringone) or nodulation of *Rhizobium* species (flavonoids) were inactive, demonstrating the specificity of the phenomenon.

Production of secondary metabolite toxins by plant pathogens is beneficial to the producing microbe in its ecological niche [83]. Tabtoxinine-β-lactam production by strains of *P. syringae* enhances the bacterium's virulence on plants and allows a tenfold increased population to develop in the plant. The mechanism by which *P. syringae* pv. "*tabaci*" protects itself against its product, tabtoxinine-β-lactam, is known [84]. This compound is an irreversible inhibitor of glutamine synthetase. Inside the pseudomonal cells, the toxin is produced as a dipeptide pretoxin, tabtoxin. During growth, the bacterial glutamine synthetase is unadenylylated and sensitive to tabtoxinine-β-lactam. However, once tabtoxin is produced, this dipeptide is hydrolyzed by a zinc-activated periplasmic aminopeptidase to tabtoxine-β-lactam, releasing serine. The serine triggers adenylylation of the pseudomonal glutamine synthetase, rendering it resistant to the inhibitor. Production of coronatine by strains of *P. syringae* – as compared to its non-producing mutant – leads to larger lesions, longer duration of lesion expansion, and higher bacterial populations of longer duration.

Xanthomonas albilineaus causes leaf scald disease of sugarcane which is characterized by chlorosis, rapid wilting, and death of the plant [85]. Chlorosis is caused by the production of the antibiotic, albicidin, by the bacterium. Albicidin kills Gram-positive and -negative bacteria and inhibits plastid DNA replication which leads to blocked chloroplast differentiation and chlorotic streaks in sugarcane. Mutants which do not form the antibiotic do not cause chlorosis [86].

A polyketide secondary metabolite, herboxidiene, produced by *Streptomyces chromofuscus*, shows potent and selective herbicidal activity [87] against weeds but not against wheat. Rice and soybean are more affected than wheat but are still relatively resistant to the microbial herbicide.

Secondary metabolites play a crucial role in the evolution and ecology of plant pathogenic fungi [88]. Some of the fungi have evolved from opportunistic

low-grade pathogens to high-grade virulent host-specialized pathogens by gaining the genetic potential to produce a toxin. This ability to produce a secondary metabolite has allowed fungi to exploit the monocultures and genetic uniformity of modern agriculture resulting in disastrous epidemics and broad destruction of crops. Fungi produce a large number of phytotoxins of varied structure such as sesquiterpenoids, sesterterpenoids, diketopiperazines, peptides, spirocyclic lactams, isocoumarins, and polyketides [89]. Production of tricothecenes by *Fusarium graminearum* is required for a high degree of plant virulence in *Fusarium* wheat head scab [90].

The AM-toxins are peptidolactones (e.g., alternariolide) produced by *Alternaria mali* which form brown necrotic spots in infected apples [91]. The phytotoxins produced by plant pathogens *Alternaria helianthi* and *Alternaria chrysanthemi* (the pyranopyrones deoxyradicinin and radicinin, respectively) are not only pathogenic to the Japanese chrysanthemum but also to fungi [92]. *Alternaria alternata* shows a specific antagonistic relationship with the spotted knapweed (*Centaurea maculosa*), prevalent in southwestern Canada and northwestern USA. The weed is inhibited only by this fungus, which produces the antibiotic maculosin (a diketopiperazine, cyclo(-L-prolyl-L-tyrosine) [93]. Interestingly, maculosin is inactive against 18 other plant species. The phytotoxin of *Rhizopus chinensis*, the causative agent of rice seedling blight, is a 16-membered macrolide antifungal antibiotic, rhizoxin [94]. The fungal pathogen responsible for onion pink root disease, *Pyrenochaeta terrestris*, produces three pyrenocines, A, B, and C. Pyrenocine A is the most phytotoxic to the onion and is the only one of the three that has marked antibacterial and antifungal activity [95].

The plant pathogenic basidiomycete, *Armillarea ostoyae*, which causes a great amount of forest damage, produces a series of toxic antibiotics when grown in the presence of plant cells (*Picea abies* callus) or with competitive fungi. The antibiotics have been identified as sesquiterpene aryl esters which have antifungal, antibacterial and phytotoxic activities [96]. One of the most pathogenic fungi in conifer forests is *Heterobasidion annosum* (syn. *Fomes annosus*) which, when grown with antagonistic fungi or plant cells, is induced to produce antibiotics against the inducing organisms [97].

With all these weapons directed by microbes against plants, the latter do not take such insults "lying down." Plants produce antibiotics after exposure to plant pathogenic microorganisms in order to protect themselves; these are called "phytoalexins" [98]. They are of low molecular weight, weakly active, and indiscriminate, i.e., they inhibit both prokaryotes and eucaryotes including higher plant cells and mammalian cells. There are approximately 100 known phytoalexins. They are not a uniform chemical class and include isoflavonoids, sesquiterpenes, diterpenes, furanoterpenoids, polyacetylenes, dihydrophenanthrenes, stilbenes, and other compopunds. Their formation is induced via invasion by fungi, bacteria, viruses, and nematodes. The compounds which are responsible for the induction are called "elicitors". The fungi respond by modifying and breaking down the phytoalexins. The phytoalexins are just a fraction of the multitude of plant secondary metabolites. Over 10,000 of these low molecular weight compounds are known but the actual numbers are probably in the

hundreds of thousands. Almost all of the known metabolites which have been tested show some antibiotic activity [99]. They are thought to function as chemical signals to protect plants against competitors, predators, and pathogens, as pollination-insuring agents and as compounds attracting biological dispersal agents [100, 101].

3.1.4 *Microorganisms vs Insects*

Certain fungi have entomopathogenic activity, infecting and killing insects via their production of secondary metabolites. One such compound is bassianolide, a cyclodepsipeptide produced by the fungus, *Beauveria bassiaria*, which elicits atonic symptoms in silkworm larvae [102]. Another pathogen, *Metarrhizium anisophae*, produces the peptidolactone toxins known as destruxins [103].

Fungi-consuming insects often avoid fungal sclerotia because of their content of secondary metabolites. Sclerotia are resistant structures which survive in soil over many years even in harsh environments. The dried fruit beetle (*Carpophilus hemipterus*) does not consume sclerotia of *Aspergillus flavus* but does eat other parts of the fungus [23]. These sclerotia contain indole diterpenoids (aflavinines) which are present only in sclerotia and inhibit feeding by the beetle. *Aspergillus nominus* produces four antibiotics (nominine, 14-hydroxypaspalinine, 14-(*N*,*N*-dimethylvalyloxy)-paspalinine and aspernomine) in sclerotia which act against the corn earworm insect, *Helicoverpazea*. Similarly, sclerotia of *Claviceps* spp. contain ergot alkaloids in high concentration which are considered to protect the sclerotia from predation. Sclerotoid ascostromata of *Eupenicillium* sp. contain insecticides that protect these fungi from insects in corn fields before they ripen and yield ascospores [104]. Corn earworm and the dried fruit beetle (*Carpophilus hemipterus*) are the insects which are inhibited by 10,23,24,25-tetrahydro 24-hydroxyaflavinine and 10,23-dihydro-24,25-dehydroaflavinine. *Eupenicillium crustaceum* ascostromata contain macrophorin-type insecticides but no aflavinines while *Eupenicillium molle* produces both types. Sclerotia of *Aspergillus* spp. also contain insecticides against these two insects. The function of the aflatoxin group of mycotoxins in aspergilli could be that of spore dispersal via an insect vector [100]. Aflatoxins are potent insecticides and *A. flavus* and *A. parasiticus*, the producing species, are pathogens of numerous insects. The fungi are brought to many plants by the insects and if the insect is killed by an aflatoxin, a massive inoculum of spores is delivered to the plant. Already a strong correlation has been established between insect damage of crops in storage and in the field and aflatoxin contamination of the crops.

Insects fight back against infecting bacteria by producing antibacterial proteins [105]. These include cecropins, attacins, defensins, lysozyme, diptericins, sarcotoxins, apidaecin, and abaecin. The molecules either cause lysis or are bacteriostatic, and also attack parasites.

Social insects appear to protect themselves by producing antibiotics [106]. Honey contains antimicrobial substances [107] and ants produce low molecular weight compounds with broad-spectrum activity [108].

3.1.5
Microorganisms vs Higher Animals

Competition may exist between microbes and large animals. Janzen [109] made a convincing argument that the reason fruits rot, seeds mold, and meats spoil is that it is "profitable" for microbes to make seeds, fresh fruit, and carcasses as objectionable as possible to large organisms in the shortest amount of time. Among their strategies is the production of secondary metabolites such as antibiotics and toxins. In agreement with this concept are the observations that livestock generally refuse to eat moldy feed and that aflatoxin is much more toxic to animals than to microorganisms. Kendrick [110] states that animals which come upon a mycotoxin-infected food will do one of four things: (i) smell the food and reject it; (ii) taste the food and reject it; (iii) eat the food, get ill, and avoid the same in the future; or (iv) eat the food and die. In each case, the fungus will be more likely to live than if it produced no mycotoxin.

Corynetoxins are produced by *Corynebacterium rathayi* and cause animal toxicity upon consumption of rye grass by animals. The disease is called "annual rye grass toxicity." The relatedness between toxins and antibiotics was emphasized by the finding that corynetoxins and tunicamycins (known antibiotics of *Streptomyces*) are identical [111].

Anguibactin, a siderophore of the fish pathogen, *Vibrio anguillarum*, is a virulence factor. When anguibactin was fed to a siderophore-deficient avirulent mutant of *V. anguillarum*, the mutant successfully established itself in the host fish [112].

Animal and plant peptides are used to defend against microbial infection [113]. They are ribosomally produced, almost always cationic, and very often amphiphilic, killing microbes by permeabilizing cell membranes. They are produced by humans, rats, rabbits, guinea pigs, mice, cattle, pigs, crabs, insects, sheep, frogs and other primitive amphibians, goats, crows, and plants. They show activities against bacteria, fungi, protozoa, and they apparently protect these higher forms of life against infection. The most well-known are the frog skin peptides, the magainins [114], which are linear peptides of approximately 20 amino acid residues. They are membrane-active, and kill by increasing permeability of prokaryotic membranes, i.e., membranes rich in acidic phospholipids but not membranes which are cholesterol-rich such as human membranes. Sharks are an example of an animal that has a primitive immunologic system yet suffers almost no infection. They apparently protect themselves by producing an antimicrobial agent in their liver, spleen, intestine, testes, etc. which is a steroid and spermidine compound with broad-spectrum activity [115].

3.2
Metal Transport Agents

Certain secondary metabolites act as metal transport agents. One group is composed of the siderophores (also known as sideramines) which function in uptake, transport, and solubilization of iron. Siderophores are complex molecules which solubilize ferric ion which has a solubility of only 10^{-18} mol/l at pH 7.4

[116]. Such siderophores have an extremely high affinity for iron ($K_d = 10^{-20}$ to 10^{-50}). The second group includes the ionophoric antibiotics which function in the transport of certain alkali-metal ions – e. g., the macrotetrolide antibiotics which enhance the potassium permeability of membranes.

Iron-transport factors in many cases are antibiotics. They are on the borderline between primary and secondary metabolites since they are usually not required for growth but do stimulate growth under iron-deficient conditions. Microorganisms have "low" and "high" affinity systems to solubilize and transport ferric iron. The high affinity systems involve siderophores. The low affinity systems allow growth in the case of a mutation abolishing siderophore production [117]. The low affinity system works unless the environment contains an iron chelator (e. g., citrate) which binds the metal and makes it unavailable to the cell; under such a condition, the siderophore stimulates growth. Over a hundred siderophores have been described. Indeed, all strains of *Streptomyces*, *Nocardia*, *Micromonospora* examined produce such compounds [118]. Antibiotic activity is due to the ability of these compounds to starve other species of iron when the latter lack the ability to take up the Fe-sideramine complex. Such antibiotics include nocardamin [119] and desferritriacetylfusigen [120]. Some workers attribute microbial virulence to the production of siderophores by pathogens and their ability to acquire iron in vivo [121]. Thus production of these iron-transfer factors may be very important for the survival of pathogenic bacteria in animals and humans [122]. Compounds specifically binding zinc and copper are also known to be produced by microorganisms.

Most living cells have a high intracellular K^+ concentration and a low Na^+ concentration whereas extracellular fluids contain high Na^+ and low K^+. To maintain a high K^+/Na^+ ratio inside cells, a mechanism must be available to bring in K^+ against a concentration gradient and keep it inside the cell. Ionophores accomplish this in microorganisms. That production of an ionophore (e. g., a macrotetralide antibiotic) can serve a survival function has been demonstrated [123]. Kanne and Zähner compared a *Streptomyces griseus* strain which produces a macrotetrolide with its non-producing mutant. In low K^+ and Na^+ media, both strains grew and exhibited identical intracellular K+ concentrations during growth. In the absence of Na^+, both strains took up K^+ from the medium. However, in the presence of Na^+, the mutant could not take up K^+. Also, when the strains were grown in high K^+ concentrations and transferred to a high Na^+, low K^+ resuspension medium, the parent took up K^+ but the mutant took up Na^+ and lost K^+. As a result of these differences, mutant growth was inhibited by a high Na^+, low K^+ environment but the antibiotic-producing parent grew well.

3.3
Microbe-Plant Symbiosis and Plant Growth Stimulants

Almost all plants depend on soil microorganisms for mineral nutrition, especially that of phosphate. The most beneficial microorganisms are those that are symbiotic with plant roots, i. e., those producing mycorrhizae, highly specialized associations between soil fungi and roots. The ectomycorrhizae, present in 3 – 5 % of plant species, are symbiotic growths of fungi on plant roots in which

the fungal symbionts penetrate intracellularly and replace partially the middle lamellae between the cortical cells of the feeder roots. The endomycorrhizae, which form on the roots of 90 % of the plant species, enter the root cells and form an external mycelium which extends into the soil [124]. Mycorrhizal roots can absorb much more phosphate than roots which have no symbiotic relationship with fungi. Mycorrhizal fungi lead to reduced damage by pathogens such as nematodes, *Fusarium*, *Pythium*, and *Phytophthera*.

Symbiosis between plants and fungi often involves antibiotics. In the case of ectomycorrhizae, the fungi produce antibiotics which protect the plant against pathogenic bacteria or fungi. One such antibacterial agent was extracted from ectomycorrhizae formed between *Cenococcum gramiforme* and white pine, red pine, and Norway spruce [125]. Two other antibiotics, diatretyne nitrile and diatretyne 3, were extracted from ectomycorrhizae formed by *Leucopaxillus cerealis* var. *piclina*; they make feeder roots resistant to the plant pathogen, *Phytophthora cinnamomi* [126].

A related type of plant-microbe interaction involves the production of plant growth stimulants by bacteria. Free-living bacteria which enhance the growth of plants by producing secondary metabolites are mainly species of *Pseudomonas*. Specific strains of the *Pseudomonas flourescens-putida* group are used as seed inoculants to promote plant growth and increase yields. They colonize plant roots of potato, sugar beet, and radish. Their growth-promoting activity is due in part to antibiotic action that deprives other bacterial species, as well as fungi, of iron. For example, they are effective biocontrol agents against Fusarium wilt and take-all diseases (caused by *F. oxysporum* F. sp. *lini* and *Gaeumannomyces graminis* var. *tritici*, respectively). Some act by producing the siderophore, ferric pseudobactin, a linear hexapeptide with the structure: L-lys-D-threo-β-OH-Asp-L-ala-D-allo-thr-L-ala-D-N6-O*H*-Orn [127]. Siderophore-negative mutants are devoid of any ability to inhibit plant pathogens [128]. In some cases, the siderophore-Fe^{3+} complex is taken up by the producing pseudomonad but in others the plant can take up the siderophore-iron complex and use it itself. Actually, plants can tolerate Fe deficiency to a much greater extent than microorganisms.

The evidence that the ability of fluorescent pseudonomads to suppress plant disease is dependent upon production of siderophores, antibiotics and HCN [129 – 136] is as follows:

1. The fluorescent siderophore can mimic the disease-suppression ability of the pseudomonad that produces it [137].
2. Siderophore-negative mutants fail to protect against disease [138, 139] or to promote plant growth under field conditions [140].
3. Antibiotic-negative rhizosphere pseudomonad mutants fail to inhibit plant pathogenic fungi [141, 142].
4. The parent culture produces its antibiotic in the plant rhizosphere [141, 143].
5. HCN-negative mutants fail to suppress plant pathogens [144].

Antibiotic-producing fluorescent *Pseudomonas* strains have been readily isolated from soils that naturally suppress diseases such as take-all (a root and crown disease) of wheat, black root rot of tobacco, and fusarium wilt of tomato [145].

Antibiotics such as pyoluteorin, pyrrolnitrin, phenazine-1-carboxylate, and 2,4-diacetylphloroglucinol are produced in the spermosphere and rhizosphere and play an important role in suppression of soil-borne plant pathogens. Suppression in a number of cases studied correlates with the production in the soil of the antibiotics.

Phenazine antibiotics production by *P. aureofaciens* is a crucial part of rhizosphere ecology and pathogen suppression by this soil-borne root-colonizing bacterium used for biological control [146]. Production of the antibiotics is the primary factor in the competitive fitness of *P. aureofaciens* in the rhizosphere and the relationships between it, the plant, and the fungal pathogens. The antibiotic, phenazine-1-carboxylate, protects wheat against take-all disease (a root and crown disease) caused by the fungus *G. graminis* var *tritici* [147]. The antibiotic is produced by *P. fluorescens* 2–79, a fluorescent pseudomonad colonizing the root system and isolated from the rhizosphere of wheat. The antibiotic inhibits the fungus in vitro and is more important than the pyoverdin siderophore produced by the same pseudomonad [132]. However, the siderophore is thought to have some role because mutants deficient in phenazine-1-carboxylate production retain some residual protection activity. Phenazine-negative mutants generated by Tn5 insertion do not inhibit the fungus in vitro and are less effective in vivo (on wheat seedlings). Cloning wild-type DNA into the mutant restored antibiotic synthesis and action in vitro and in vivo. The antibiotic could be isolated from the rhizosphere of the wheat colonized by strain 2–79 and disease suppression was correlated with its presence [141]. The ability of *P. fluorescens* and *P. aureofaciens* to produce phenazine antibiotics is not only responsible for protection of wheat roots but also aids in survival of the producing bacteria in soil and in the wheat rhizosphere [148]. Phenazine-negative mutants survive poorly due to a decreased ability to compete with the resident microflora. In addition to phenazine-1-carboxylate, *P. aureofaciens* produces 2-hydroxyphenazine-1-carboxylate and 2-hydroxyphenazine, which are also active in plant protection [149]. Another antibiotic protecting wheat against take-all disease is 2,4-diacetylphloroglucinol (DAPG) which is produced by strain 9287 of *P. aureofaciens*. Nonproducing mutants fail to protect, and such mutants, when transformed with the missing gene, produce antibiotic and protect wheat [150]. The frequency of DAPG-producing cells is high in soils suppressing take-all and is undetectable or at most 2.5 % of the above frequency in soils conducive to take-all disease of wheat.

The production of the antibiotic oomycin A by *P. fluorescens* HV37a protects cotton seedlings from *Pythium ultimum* which causes preemergence root infections [151].The disease is known as damping off disease. Mutation of the fungus to non-production markedly lowers the ability to control the disease [152]. Damping off of cotton and other plants is also caused by *Rhizoctonia solani*. In this case, protection is provided via pyrrolnitrin production by *P. fluorescens* BL915. Protection is ineffective with non-producing mutants unless they first receive wildtype DNA [153]. Cloning such DNA into natural non-producing strains of *P. fluorescens* also conveys pyrrolnitrin production and ability to protect plants. The production strain and non-producing wildtypes are all inhabitants of cotton roots. Two siderophores produced by the plant-growth

promoting rhizobacterium *P. aeruginosa* 7NSK2, i. e., pyochelin and pyoverdin, are involved in suppression of damping-off disease of tomato caused by *Pythium* [154]. Either one or the other siderophore serves as the effective agent, i. e., if one is not produced, the other serves to protect. A third siderophore, salicylic acid, appears to provide some protection in the absence of pyochelin and pyoverdin. In the case of *Pseudomonas* sp. N2130, this fluorescent rhizosphere bacterium produces two iron-regulated secondary metabolites, one a siderophore, the other a non-siderophore. Only the non-siderophore is an antifungal agent [155].

Bacillus cereus UW85 protects against damping off disease of alfalfa seedlings caused by *Phytophthera medicaginis*. It also protects tobacco seedlings from *Phytophthera nicotianae*, cucumbers from *Pythium aphanidermatum* rot, peanuts from *Sclerotinia minor*, and enhances nodulation of soybeans by changing distribution of bacteria on roots [156]. Two extracellular antibiotics are responsible for protection against damping off: (i) zwittermycin A, a linear aminopolyol and (ii) antibiotic B, an aminoglycoside antibiotic containing a disaccharide. Zwittermycin A inhibits elongation of the germ tubes of *P. medicaginis* tubes and antibiotic B causes the tubes to swell. In low- and non-producing mutants of *B. cereus*, antibiotic production and disease suppression are quantitatively correlated. When plants are inoculated with an inactive mutant, disease occurs but this can be prevented by addition of either antibiotic. In a survey of 96 strains isolated around the world, isolates producing either zwittermycin A or antibiotic B more effectively controlled the alfalfa disease than strains producing neither antibiotic [157]. Antibiotic production by *Bacillus subtilis* CL27 is the mechanism of its biocontrol of *Botrytis cinerea* damping off disease of cabbage seedlings [158]. The *Bacillus* strain, isolated from Brassica leaves, produces two peptide antibiotics and one non-peptide antibiotic. A mutant lacking ability to produce the latter is less active in vivo and a mutant lacking the ability to produce all three antibiotics is inactive in vivo.

Control of rhizoctonia root rot of pea by inoculated *Streptomyces hygroscopicus* var. *geldanus* is due to production of geldanomycin [159]. The antibiotic was extracted from soil and shown to be active against *Rhizoctonia solani*. Addition of geldanomycin itself to soil also controls disease. Potato scab disease is caused by *Streptomyces scabies* and biocontrol of the disease can be carried out with *Streptomyces diastatochromogenes* isolated from potato. The latter produces an antibiotic that appears to be involved in the mechanism of its biocontrol [160]. Interestingly, the antibiotic inhibits the pathogenic *S. scabies* strains but not other species of *Streptomyces* and other bacteria.

Another important factor in the pathogenic or beneficial relationships between bacteria and plants is the ability of plant-associated bacteria to produce the phytohormone (auxin) indole-3-acetic acid [161]. The bacteria are species of *Pseudomonas*, *Agrobacterium*, *Rhizobium*, *Bradyrhizobium*, and *Azospirillum*.

Two secondary metabolites, altechromones A and B, produced by *Alternaria* sp. isolated from oats are plant-growth stimulators [162]. *Taxus wallachiana*, the Nepalese yew tree, has an endophytic fungus, *Phoma* sp. living in the intercellular spaces of its bark tissue [163]. The relationship is thought to be mutualistic in which the plant provides nutrients to the fungus and the fungus protects

the plant from plant pathogens. *Phoma* sp. produces two antibiotics, altersolanol A and 2-hydroxy-6-methylbenzoic acid.

3.4
Microbe-Nematode Symbiosis

Antibiotics play a role in the symbiosis between the bacteria of the genus *Xenorhabdus* and nematodes parasitic to insects [164]. Each nematode species, members of the *Heterorhabditidae* and *Steinernematidae*, is associated with a single bacterial species of *Xenorhabdus* [165]. The bacteria live in the gut of the nematode. When the nematode finds an insect host, it enters and when in the insect gut it releases bacteria which kill the insect, allowing the nematode to complete its life cycle. Without the bacteria, no killing of the insect occurs. The bacteria produce antibiotics to keep the insect from being attacked by putrefying bacteria. Two groups of antibiotics have been isolated from two of the bacteria. One group is represented by tryptophan derivatives and the other by 4-ethyl- and 4-isopropyl-3,5-dihydroxy-*trans*-stilbenes [164]. The antibiotic produced by *Xenorhabdus luminescens*, the bacterial symbiont of several insect-parasitic nematodes of the genus *Heterorhabditis*, has been identified as the hydroxystilbene derivative 3,5-dihydroxy-4-isopropylstilbene [166]. Other strains of *X. luminescens* produce indole antibiotics.

3.5
Microbe-Insect Symbiosis

Symbiosis between intracellular microorganisms and insects involves antibiotics. The brown planthopper, *Nilaparavata lugens*, contains at least two microbial symbionts and lives on the rice plant. One intracellular bacterium is *Bacillus* sp. which produces polymyxin M [167]. Another is *Enterobacter* sp. producing a peptide antibiotic selective against *Xanthomonas campestris* var *oryzae*, the white blight pathogen of rice [168]. The intracellular bacteria increase their survival chances via antibiotic production to protect the insect from invasion by microorganisms and to control competition by bacterial rice pathogens.

3.6
Microbe-Higher Animal Symbiosis

Antibiotic production by the commensal bacterium, *Alteromonas* sp., is responsible for protection of embryos of the shrimp, *Palaemon macrodactylus*, from the pathogenic fungus *Lagenidium callinectes* [169]. The antifungal agent which mediates protection is 2,3-indolinedione (isatin). Similarly, eggs of *Homarus americanus* (the American lobster) are covered with a bacterium that produces tyrosol [2-(*p*-hydroxyphenyl) ethanol], an antimicrobial agent [170]. The filamentous tropical cyanobacterium, *Microcoleus lyngbyaceus*, contains four specific bacteria on its surface, all of which produce quinone 34, an antibacterial and antifungal compound [170]. In this regard, a number of antibiotics which

were thought to be produced by sponges are now considered to be made instead by bacteria living in and/or on these higher forms [170–172].

3.7 Sex Hormones

Many secondary metabolites function as sexual hormones, especially in fungi [173, 174]. The most well known are the trisporic acids, which are metabolites of *Mucorales*. When vegetative hyphae of the two mating types of these heterothallic organisms approach one another, they form zygophores (sexual hyphae). The trisporic acids, factors that induce zygophore formation, are formed from mevalonic acid in a secondary metabolic pathway of which the early steps are present in both (+) and (–) sexes. However, distinct late steps are missing in the individual sexes and thus both strains must be present and in contact to complete the pathway to the trisporic acids. In *Gibberela zeae* (*Fusarium roseum*), the secondary metabolite, zearalenone, is a regulator of sexual reproduction [175]. The secondary metabolite, sirenin, is involved in sexual reproduction in *Allomyces*, a phycomycete. It acts as a chemotaxic hormone which brings together uniflagellate motile male and female gametes. Sirenin, a sesquiterpene diol made by female gametangia and gametes, is extremely active, the process requiring less than 0.1 ng/ml for activity [176]. In the phycomycete *Achyla*, antheridol, a steroidal secondary metabolite, is produced by vegetative female mycelia and initiates the formation of male gametangia. The compound is active at concentrations as low as 10^{-11} mol/l [177]. The male ganetangia produce another secondary metabolite (hormone B) which leads to oogonia formation in female mycelia. *Tremella mesenterica*, a jelly fungus of the heterobasidomycete group, produces the peptide tremerogen A-10 which induces germ tubes in mating type a [178]. A compound inducing sexual development in *Aspergillus nidulans* has been isolated [179]. Crude preparations containing the factor (called psi) are active at levels as low as 50 ng per test.

Male *Aphomia sociella* L, the bumble bee waxmoth, contains a sex pheromone in its wing gland, the major part of which is an R(–)mellein (= ochracin). The compound, which evokes searching behavior in females, is produced by a mold, *Aspergillus ochraceus*, found in the intestine of the last-instar larvae and in the bumble bee nest [180]. Apparently such insect-fungus relationships are widespread.

Substances I_A, I_B, and I_C are peptidic sexual agglutination factors of *Saccharomyces cerevisiae* [181]. Rhodotorucine A is a peptide produced by type A cells of *Rhodospridium toruloides* which induces mating tube formation in yeast of mating type a [182]. A bacterial sex pheromone, called cPD1, has been isolated from *Streptococcus faecalis*. Its structure is phe-leu-val-met-phe-leu-ser-gly [183]. Competence in *Streptococcus pneumoniae* is induced by a heptadecapeptide, *H*-Glu-Met-Arg-Leu-Ser-Lys-Phe-Phe-Arg-Asp-Phe-Ile-Leu-Glu-Arg-Lys-Lys-OH which is destroyed by a protease [184].

Microbial secondary metabolites can exert regulation of cellular activities in higher organisms [185]. It has been hypothesized that cell-to-cell communication first evolved in unicellular organisms, long before the appearance of specialized

cells of vertebrates (glands, neurons, immune cells, blood cells) [186]. Thus hormones, neuropeptides, biological response modifiers, and their receptors may have been first made by microorganisms. Indeed, steroid fungal sex hormones and mammalian sex hormones are similar in structure.

3.8 Effectors of Differentiation

Development is composed of two phenomena, growth and differentiation. The latter is the progressive diversification of structure and function of cells in an organism or the acquisition of differences during development [47]. Differentiation encompasses both morphological differentiation (morphogenesis) and chemical differentiation (secondary metabolism). Secondary metabolites produced by chemical differentiation processes also function in morphological and chemical differentiation.

3.8.1 *Sporulation*

Of the various functions postulated for secondary metabolites, the one which has received the most attention is the view that these compounds, especially antibiotics, are important in the transition from vegetative cells to spores. The following observations have made this hypothesis attractive:

1. Practically all sporulating microorganisms produce antibiotics.
2. Antibiotics are frequently inhibitory to vegetative growth of their producers at concentrations produced during sporulation.
3. Production of peptide antibiotics usually begins at the late-exponential phase of growth and continues during the early stages of the sporulation process in bacilli.
4. Sporulation and antibiotic synthesis are induced by depletion of some essential nutrient.
5. There are genetic links between the synthesis of antibiotics and the formation of spores; revertants, transductants, and transformants of stage 0 asporogenous mutants, restored in their ability to sporulate, regain the ability to synthesize antibiotic while conditional asporogenous mutants fail to produce antibiotic at the non-permissive temperature.
6. Physiological correlations also favor a relationship between the production of an antibiotic and spores. As an example, inhibitors of sporulation inhibit antibiotic synthesis. Furthermore, both processes are repressed by nutrients including glucose. Concentrations of manganese ion of at least two orders of magnitude higher than that required for normal cellular growth are needed for sporulation and antibiotic synthesis by certain species of *Bacillus*.
7. There appears to be a direct relationship between formation of ergot alkaloids and conidiation in *Claviceps purpurea* [187].

Much enthusiasm in favor of an *obligatory* function of antibiotics in sporulation derived from early work [188] which reported that cessation of exponential

vegetative growth of *Bacillus brevis* ATCC 8185 is accompanied by tyrothricin synthesis and a sharp decline of net RNA synthesis. It was also stated that both antibiotic components of tyrothricin (tyrocidine and linear gramicidin) are capable of inhibiting purified *B. brevis* RNA polymerase. The view was advanced that antibiotics regulate transcription during the transition from vegetative growth to sporulation by selectively terminating the expression of vegetative genes. Although the inhibition of RNA polymerase by tyrocidine and linear gramicidin was confirmed [189, 190], an obligatory relationship between production of the two antibiotics, inhibition of RNA synthesis, and sporulation has yet to be established. Even in other studies [191], in which tyrothricin was found to stimulate sporulation when early log phase cultures were incubated in a glycerol medium lacking nitrogen, this addition stimulated RNA synthesis rather than inhibiting it.

Despite the apparent connections between formation of antibiotics and spores, it has become clear that antibiotic production is not obligatory for spore formation [192]. The most damaging evidence to the antibiotic-spore obligatory hypothesis is the existence of mutants which form no antibiotic but still sporulate. Such mutants have been found in the cases of bacitracin (*Bacillus licheniformis*), mycobacillin (*Bacillus subtilis*), linear gramicidin (*B. brevis*), tyrocidine (*B. brevis*), gramicidin S (*B. brevis*), oxytetracycline (*Streptomyces rimosus*), streptomycin (*S. griseus*), methylenomycin A (*Streptomyces coelicolor*), and patulin (*Penicillium urticae*).

When little to no evidence could be obtained to support the hypothesis that antibiotics are necessary to form spores, further studies [193] focused on the quality of spores produced without concurrent formation of antibiotics. A mutant was obtained of the tyrothricin-producing *B. brevis* ATCC 8185 which produced normal levels of tyrocidine and spores but did not produce linear gramicidin. The spores were claimed to be less heat-resistant than normal but other workers were unable to confirm these findings [194]. Similarly, mutants producing linear gramicidin but not tyrocidine formed spores of normal quality [195]. Studying the *B. brevis* strain which produces gramicidin S, it was reported [196] that non-producing mutants form heat-sensitive spores but again this was not confirmed [194, 197].

Although antibiotic production is not obligatory for sporulation, it may stimulate the sporulation process [198]. Transfer of exponential phase populations of *B. brevis* ATCC 8185 (the tyrothricin producer) into a nitrogen-free medium stops growth and restricts sporulation. Supplementation of the medium with tyrocidine induces sporulation. Tyrocidine cleaved by a proteolytic enzyme, its component amino acids, and gramicidin S are all inactive. This indicates that the tyrocidine component of tyrothricin is an inducer of sporulation. Sporulation-associated events of *B. brevis* ATCC 8185 were turned on by linear gramicidin addition when nitrogen limitation was made more severe, i. e., by washing the cells before resuspension in the absence of nitrogen source [199]. In this case, the production of extracellular protease, RNA, dipicolinate, and tyrocidine itself was also stimulated. Addition of linear gramicidin also brought about a severe depletion of intracellular ATP. Non-ionophoric analogs of linear gramicidin did not exert the sporulation effect.

Bacilysin, a dipeptide antibiotic, may play a stimulatory role in the sporulation of *B. subtilis* [200]. A bacilysin-negative strain, NG79, was found to be oligosporogenous, the spores to be sensitive to heat, chloroform, and lysozyme, and deficient in dipicolinic acid. When the strain was transduced to bacilysin production, the above characteristics also returned to wild-type status. Addition of bacilysin to the mutant increased sporulation resistance, and dipicolinic acid content. The concept that bacilysin plays a role in sporulation of *B. subtilis* is supported by the observation that interference in bacilysin production by addition of ammonium ions, Casamino acids, or L-alanine results in blockage of sporulation [201]. Although glucose interferes with sporulation but not bacilysin formation, and decoyinine induces sporulation with no effect on bacilysin production, these observations can be explained as effects on sporulation independent of those on bacilysin synthesis.

An extracellular peptide, EDF-A is required for sporulation of *B. subtilis* [202]. Its production is cell-density dependent. Thus, dilute bacterial suspensions sporulate poorly when decoyinine is added or the population is shifted down, unless EDF-A, present in the extracellular medium of high-density preparation, is added. The pheromone is destroyed by pronase and is dialyzable and heat-resistant. EDF-A production is defective in *spoOA* or *spoOB* mutants. Mutations in *abrB*, which suppress many of the pleiotropic phenotypes in *spoOA* mutants – except sporulation – restore production of EDF-AA.

Sporulation inducers are also known in the actinomycetes. One such compound is the antibiotic pamamycin produced by *Streptomyces alboniger*. Pamamycin inhibits *Staphylococcus aureus* by interfering with uptake of inorganic phosphate and nucleosides [203]. In the producing culture, the antibiotic stimulates the formation of aerial mycelia and thus that of conidia [204]. Pamamycin has been found to be a family of eight homologous compounds varying in size from 593 Da to 691 Da [205]. The homolog of molecular weight 607 is active as an antibiotic against fungi and bacteria. It induces aerial mycelium formation on agar in a negative-aerial mycelium mutant at 0.1 μg/paper disk and inhibits vegetative growth of the producing *S. alboniger* at 10 μg/disk. Its structure is that of a macrolide and it has the activity of an anion transfer agent. Chou and Pogell [206] had reported its action to be that of an inhibitor of phosphate uptake. Some homologs of pamamycin-607, produced by *S. alboniger* along with pamamycin-607, retain the ability to inhibit growth of the aerial mycelium-deficient mutant but do not induce aerial mycelium formation in the mutant [207].

A-Factor in *S. griseus* induces formation of streptomycin, aerial mycelia, and conidia [208]. Many such γ-butyrolactones are produced by actinomycetes. In the producer of leukaemomycin, an anthracycline antibiotic, the structure differs slightly from that of A-Factor [209]. Other inducers of aerial mycelium formation include toxopyrimidine (2-methyl-4-amino-5-hydroxymethylpyrimidine) in *Streptoverticillium* species [210] and borrelidin, a macrolide antibiotic, produced by *Streptomyces parvulus*; borrelidin acts as inducer for *Streptomyces tendae bld* mutants [211]. Hormaomycin, a peptide lactone antibiotic produced by *S. griseoflavus*, induces aerial mycelium and antibiotic formation in other streptomycetes [212]. *Streptomyces* sp. produces basidifferquinone, which induces fruiting body formation in the basidiomycetous fungus, *Favolus arcularius* [213].

Factors inducing sporulation have also been isolated from fungi. A sesquiterpenoid with an eremophilane skeleton was found to be a sporogenic factor (sporogen-A01) produced by *Aspergillus oryzae* [214]. Five cerebrosides isolated from *Schizophyllum commune* induce fruiting body formation in the same organism. All five were identified, the major component being (4*E*,8*E*)-*N*-2′-hydroxyhexadecanoyl-1–0-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine [215]. An antifungal agent, lunatoic acid, produced by *Cochliobolus lunatus* is an inducer of chlamydospore formation [216].

Carbazomycinal and 6-methylcarbazomycinal, inhibitors of aerial mycelium formation, are produced by *Streptoverticillium* sp. [217].

3.8.2 *Germination of Spores*

The close relationship between sporulation and antibiotic formation suggests that certain secondary metabolites involved in germination might be produced during sporulation and that the formation of these compounds and spores could be regulated by a common mechanism or by similar mechanisms.

A number of secondary metabolites are involved in maintaining spore dormancy in fungi. One example of these germination inhibitors is discadenine [3-(3-amino-3-carboxypropyl)-6-(3-methyl-2-butenylamino)purine] in *Dictyostelium discoideum*, *Dictyostelium purpureum*, and *Dictyostelium mucoroides* [218]; this compound is made from 5′-AMP [219]. Their function appears to be that of inhibiting germination under densely crowded conditions, and they are extremely potent secondary metabolites. The auto-inhibitor of uredospore germination in *Puccinia coronata* var *avenae* (oat crown rust fungus) is methyl-*cis*-3,4-dimethoxycinnamate [220]. The auto-inhibitor of conidial germination in *Colletotrichum graminicola* is the secondary metabolite, mycosporine alanine [221].

Germination inhibitors have also been found in actinomycetes. Germicidin [6-(2-butyl)-3-ethyl-4-hydroxy-2-pyrone], produced by *Streptomyces viridochromogenes*, is a weak antibiotic uncoupling respiration from ATP production until it is excreted during germination [222, 223]. The antibiotic is a specific inhibitor of an ATP synthase and appears to be responsible for maintaining dormancy of the spores. It blocks ATP synthesis in the spores and thus uncouples glucose oxidation from ATP synthesis. Upon addition of the germinating agent Ca^{++}, the inhibitor is excreted from the spore, the ATP synthase is activated by the Ca^{++}, ATP is synthesized as glucose is oxidized, and germination ensues.

Considerable evidence has been obtained indicating that gramicidin S (GS) is an inhibitor of the phase of spore germination known as “outgrowth” in *B. brevis* [196, 197, 199, 224, 225]. The cumulative observations are as follows:

1. Initiation of germination (i. e., the darkening of spores under phase microscopy) is similar in the parent and GS-negative mutants.
2. GS-negative mutant spores outgrow in 1 – 2 h whereas parental spores require 6 – 10 h. The delay in the parent is dependent on the concentration of spores and hence the concentration of GS.

3. Addition of GS to mutant spores delays their outgrowth so that they now behave like parental spores; the extent of the delay is concentration-dependent and time-dependent.
4. Preparation of parental spores on media supporting poor GS production results in spores which outgrow as rapidly as mutant spores.
5. Removal of GS from parental spores by extraction allows them to outgrow rapidly.
6. Addition of the extract to mutant spores delays their outgrowth.
7. Exogenous GS hydrolyzed by a protease does not delay outgrowth of mutant spores. Parental spores treated with the protease outgrow rapidly.
8. Exponential growth is not inhibited by GS.
9. A mixture of parental spores and mutant spores shows parental behavior, so that the mixture is delayed in outgrowth. This indicates that some of the GS externally bound to parental spores is released into the medium. This release could act as a method of communication by which a spore detects crowded conditions.
10. Uptake of alanine and uridine into spores and respiration are inhibited by GS [199, 226, 227].

Lobareva et al. [228] provided evidence that *B. brevis* excretes over 90 % of its GS which is then bound to the outside of the spores. The bound GS can be removed (with difficulty) by water or buffer, the ease of which is dependent on the presence or absence of detergents and also pH. They showed that it is not merely a case of insoluble GS in suspension but that soluble GS binds to the cells. They believe this excretion process is the way *B. brevis* protects itself against GS uncoupling of electron transport and phosphorylation, i. e., energy production.

GS antibiotic activity appears to be due to its surface-activity: interaction with artificial lipid bilayers, and with mitochondrial and bacterial bilayers and membranes. There is an electrostatic interaction between membrane phospholipids and GS causing a phase separation of negatively charged phospholipids from other lipids, leading to a disturbance of the membrane's osmotic barrier. It is possible that this effect is responsible for GS's ability to inhibit respiration and uptake (of uridine and alanine) during germination of spores of the producing organism and to delay spore outgrowth. GS does not inhibit growth of vegetative cells of the producer *B. brevis* ATCC 9999 or of *E. coli*, but it does inhibit the growth of vegetative cells of *B. subtilis*. However, alanine and uridine uptake into membrane vesicles from all three organisms is inhibited [229]. It is unclear why vegetative cells of the GS-producer are resistant to GS. Although Danders et al. [229] proposed that this is due to impermeability, Frangou-Lazaridis and Seddon [230] pointed out that exogenous GS added to vegetative cells is incorporated into the resulting spores [225] and thus is able to enter vegetative cells. It is of interest that tyrocidine, which has a structure similar to GS, also shows antibiotic action against *B. subtilis*, inhibition of active transport in the three species mentioned above, and delay of spore outgrowth of the GS-producing species, all to a lesser degree than GS [229]. Danders and coworkers [226, 229] reported that one difference between GS and tyrocidine is that GS does not bind to DNA and inhibit RNA polymerase whereas tyrocidine does. However, there is

a serious disagreement between groups on this point. Frangou-Lazaridis and Seddon [230] found that RNA polymerase from the producing strain is inhibited by GS. They reported that addition of GS to *B. brevis* Nagano or its GS-negative mutant E-1 has no effect on growth or sporulation when added during or after the logarithmic phase of growth yet it can permeate the cells. No effect was seen on incorporation of labeled lysine, thymidine, or uracil by intact cells or on transcription by permeabilized vegetative cells although they were inhibited by rifamycin and actinomycin. However, RNA polymerase in vitro was strongly inhibited by GS. Frangou-Lazardis and Seddon [230] concluded that transcription is the sensitive step during germination outgrowth. Inhibition was thought to be due to GS complexing with DNA, not with the enzyme. They suggest that DNA and GS are prevented from interacting during growth or that vegetative DNA is in a conformational state that is not vulnerable to GS.

Irrespective of the mode of action of GS in inhibiting germination outgrowth, there are several other questions about this phenomenon which need to be addressed.

First, what would be the value to the producing organism of inhibiting germination in the outgrowth stage during which spores of bacilli are thought to have lost their resistance to factors such as heat? Would not inhibition at this stage make more spores more susceptible to attack by other organisms and would not rapid outgrowers (e.g., non-producing mutants) be selected? It turns out, however, that initiated (i.e., phase-dark) spores of GS-producing *B. brevis* are still resistant to heat, starvation, solvents, and even to sonication [231]. It is also of interest that studies on survival of *Bacillus thuringiensis* spores in the soil have revealed that rapid germination ability of spores in soil confers no survival advantage [232].

It appears that endogenous GS is the basis of the hydrophobicity of dormant or initiated *B. brevis* spores. After outgrowth ceases, the resulting vegetative cells are hydrophilic [233]. Since water-insoluble organic matter constitutes the chief source of soil nutrients [63], it is quite possible that the hydrophobicity of *B. brevis* spores and initiated spores aids in their search for nutrients to insure vegetative growth after germination. If no nutrients are found, it is possible that initiated spores can develop back into normal spores by microcycle sporulation [234] which may have a role in nature [235].

A second question involves the mechanism by which the outgrowing spores recover from GS inhibition and finally develop into vegetative cells. One possibility is destruction of GS towards the end of the outgrowth stage. *B. brevis* ATCC 9999 produces an intracellular serine protease [236–238] despite earlier claims that it does not [239]. This type of enzyme is generally considered to be necessary for sporulation of bacilli. The *B. brevis* enzyme has the ability to cleave GS between val and orn residues [237]. No extracellular proteases are produced by ATCC 9999, a situation very different from most bacilli. Although it would appear that the intracellular enzyme might function to destroy GS and allow vegetative growth from outgrown spores (e.g., it was claimed [240] that GS is destroyed at that time), data indicate that GS is not destroyed as the outgrowing spores develop into vegetative cells [241–243]. Furthermore, the recovery is not due to selection of spores whose outgrowth is resistant to GS [241]. Another pos-

sibility is that GS kills outgrowing spores and the delay in outgrowth is merely the time required by a small population of unkilled spores to germinate and become vegetative cells. Although the finding that GS kills a large proportion of outgrowing spores [244] has been confirmed, the same residual fraction of survivors is seen despite increases in the GS concentration [243]. The contrast between the failure of increased concentrations of GS to affect killing can be contrasted with the increasing delay in outgrowth caused by the increased concentration and makes unlikely a connection between the degree of killing and the increasing delay of outgrowth caused by raising the concentration. At this point, it appears that GS, because of its inhibition of oxidative phosphorylation, transport and/or transcription slows down – but does not totally inhibit – the macromolecular processes of outgrowth, until a point is reached where all the outgrown spores have the proper machinery to differentiate into vegetative cells. During this process, GS is excreted into the extracellular medium [225, 227].

It is thus probable that GS serves the initiated spore as a means of sensing a high population density and preventing vegetative growth until there is a lower density of *B. brevis* spores with which to compete for nutrients. However, proof of such a hypotheses will require experimentation of an ecological nature. Alternative hypotheses might be that GS in and on the dormant and initiated spores protect them from consumption by amoebae or that GS excretion during germination initiation and outgrowth eliminates microbial competitors in the environment. Another possibility is that the delay in outgrowth and death of a part of the outgrowing spore population is merely "the price the strain must pay" for such protection.

3.8.3
Other Relationships Between Differentiation and Secondary Metabolites

Another relationship between secondary metabolites and differentiation is stimulation of germination. Germination stimulators in rust fungi include nonanal and 6-methylhept-5-en-2-one which work on uredospores [245]. In addition to producing extracellular ferric ion-transport and solubilizing factors (siderophores), fungi produce cell-bound siderophores which are involved in conidial germination [246]. A siderophore is required for conidial germination in *Neurospora crassa* [247]. These siderophores are considered to be iron storage forms in fungal spores that are analogous to the ferritins of animals and the phytoferritins of plants [248].

Cyclic AMP (c-AMP) is a secondary metabolite in the slime mold, *Dictyostelium discoideum*. It is the chemotactic agent which, after initiation of development by starvation, attracts the amoebae-type cells and aggregates them to form the elongated, multi-cellular "slug" structures. Each cell of the slug differentiates into either a stalk cell or a spore of the fruiting body. Differentiation depends on c-AMP plus a low molecular weight factor known as differentiation inducing factor (DIF). A high ratio of DIF to c-AMP appears to produce a stalk cell whereas, a low ratio produces a spore [249].

Campbell and associates [49] have made interesting observations on secondary metabolite production by colonies growing on solid media which further

implicate these molecules in differentiation. In general, they find particular secondary metabolites to be produced only by certain differentiating structures. In *Penicillium patulum*, 6-methylsalicylic acid (6-MSA) is produced only after aerial mycelia are formed. The same is true in *Penicillium brevicompactum*, with respect to formation of mycophenolic acid, brevianamides A and B, asperphenamate, and ergosterol. Asperphenamate and ergosterol are the first to be formed, followed by mycophenolic acid, all three being made before the appearence of conidial heads. The brevianamides are produced only after the conidial heads appear and during condidiation. In *P. patulum*, for example, 6-MSA synthesis begins prior to the formation of conidial heads. While conidiophores of *P. brevicompactum* are producing brevianamides, they rotate when exposed to UV or visible light; no rotation occurs if brevianamides are not present. Upon rotation, water is pumped up the conidiophore. Therefore, it has been proposed that brevianamides are involved in water translocation from the substrate up the conidiophore into the penicillus.

3.9 Miscellaneous Functions

Prodigiosin may function in air dispersal of *Serratia marcescens* [250]. The pigmented strain is much more able than the non-pigmented strain to adsorb onto air bubbles and enrich the drops formed on breakage of the bubbles. Also prodigiosin makes hydrophobic the normally hydrophilic cells. The pigment itself is hydrophobic.

Astaxanthin, a carotenoid secondary metabolite of the basidiomycetous yeast, *Phaffia rhodozyma*, protects the organism against killing by singlet oxygen [251] and provides a selective advantage against albino mutants. The natural habitat of the yeast is sap flux of the birch tree, *Betula*. The tree contains an unidentified compound which catalyzes formation of singlet oxygen when exposed to UV light. Singlet oxygen induces formation of astaxanthin and the latter causes negative feedback regulation of its synthesis.

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Development of Applied Microbiology to Modern Biotechnology in Japan

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Development of modern biotechnology in Japan is characterized by unique contributions from applied microbiology and bioindustry. This review tries to summarize these original contributions with special emphasis on industrial production of useful substances by microorganisms. In the first part, development of applied microbiology and bioindustry in the last half of the twentieth century is summarized with a brief overview of the traditional background. In the second part, recent progress is reviewed with citation of typical achievements in biotechnology, applied enzymology, secondary metabolites, genetic engineering, and screening of microbial diversity, respectively.

Keywords. Screening, Bioindustry, Applied enzymes, Secondary metabolites, Genetic engineering, Microbial diversity

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1 Introduction

Science and technology are international but their development can be affected by regional characteristics. This aspect is observed with development of biotechnology in Japan, which is characterized by unique contributions from applied microbiology and bioindustry. During the second half of the twentieth century, bioindustry in Japan has made rapid progress by developing many innovative processes for microbial production of a variety of useful substances including foodstuff additives, enzymes, pharmaceuticals, pesticides, and other chemicals. Applied microbiology played crucial roles in this development especially through discovery of novel microbial functions by means of extensive screening. Bioindustry also played important roles for industrialization of new biotechnology as manifested in the production of heterologous proteins by recombinant DNA technology. Experiences with the microbial diversity as well as basic understandings on the molecular mechanisms in microbial cells accumulated during these decades led to transformation of applied microbiology into a characteristic complex of modern biotechnology. This review deals with personal overview about a brief history of this development along with its latest achievements.

2 Historical Overview of Applied Microbiology in Japan

2.1 Traditional Background

Japan has a long tradition in the fermentation industry to produce rice wine "sake" and a variety of fermented foodstuffs such as fermented soy sauce "shoyu". Before introduction of modern science and technology at the end of the last century, engineer's guilds in the brewing manufacturers had established a sophisticated system of rational technologies, even empirically. The best example is the sake brewing process, in which saccharification of rice starch by amylases

from a fungus *Aspergillus oryzae* and ethanol fermentation by yeast *Saccharomyces cerevisiae* proceed in parallel in a fermenter. Fine techniques to control microflora enabled stable operation of this complex process to produce ethanol at the world-highest concentration as high as 20% with an inherent flavor of high quality.

Interestingly enough, the first industrial application of microbial enzymes started in the USA in 1894 was a direct descendant of the sake brewing technology, which was made by a Japanese scientist, Jokichi Takamine, in Peoria, Illinois. He modified the traditional solid-state culture process of *A. oryzae* for industrial production of a mixed enzyme preparation "Taka-Diastase" containing amylases and other extracellular enzymes, and applied the preparation first to the production of alcoholic beverage from grains and then to the treatment of dyspepsia or indigestion. This was a pioneering enterprise for application of microbial enzymes, whose lineage can still be traced in several companies in the USA and Japan. It also heralded the following general trend to replace the enzyme resources from higher plants or animals to microorganisms.

An event that exerted strong influence on the later development of bioprocesses in Japan is the discovery of monosodium L-glutamate as a flavor enhancer of food in 1908. Kikunae Ikeda, Professor of the University of Tokyo, was interested in dried kelp, a traditional seasoning material for cooking in Japan, and succeeded in identifying the amino acid as the essence of its flavor. Ajinomoto (meaning Essence of Flavor) Co. started its industrial production by acid hydrolysis of wheat gluten in 1909, and thus opened a big market of food flavor. This original invention prepared the basis for the later innovation of the amino acid process.

Success in developing unique technologies through screening of new microbial functions may be one of the major features of applied microbiology in Japan. Kin-ichiro Sakaguchi (1897 – 1994), a leader of applied microbiology from the beginning, once made a short remark that has been passed among his students during these decades: "I have never been disappointed upon asking microorganisms for whatever I wanted." As an embodiment of his statement, a memorial stone to commemorate the contribution of microorganisms to human beings is situated in front of an old temple in the historic capital, Kyoto. Such an atmosphere may also be seen as a traditional background, which has encouraged researchers engaging in screening projects with high risk.

2.2 Launching the Modern Bioindustry with Antibiotics

Research and development of antibiotics played an important role in constructing modern bioindustries from the ruins after the Second World War. The first scientific information on penicillin described in a medical journal reached Japan during the war in 1943, which was delivered from Germany by a Japanese navy submarine. The penicillin research committee consisting of multi-disciplinary researchers was quickly organized and succeeded in realizing small-scale production of penicillin by surface culture by 1945. Real potential of the research system was expressed after the war upon the generous introduction of *Penicillium*

chrysogenum strain Q176 from the USA in 1946. The research association reorganized by incorporating industrial members took a principal role in research and development, and achieved stable industrial production of penicillin by submerged culture within a few years. A similar strategy was once again adopted to develop streptomycin production to meet urgent demand to cure tuberculosis patients, the death rate of which exceeded 180 per 100,000 persons in 1948, and succeeded in much faster development than the former case. Close association between academia and industries in the field of applied microbiology has originated during these developmental days.

Then discovery of a number of new antibiotics of practical usefulness, such as the first 16-membered macrolide antibiotic leucomycin (1953), mitomycin C (1956), and kanamycin (1957), followed soon after. Those are the indications that the principal methodologies for research and development of antibiotics, especially random screening of new antibiotic producers from nature, firmly took root in many research groups and companies. Among them, Umezawa and his group, first at the University of Tokyo and later at his own Institute of Microbial Chemistry, played a leading role. Kanamycin discovered by his group was very effective against multi-drug-resistant pathogens and tuberculous bacilli [1]. Later, bacteria resistant to kanamycin appeared, then Umezawa revealed a resistance mechanism due to an inactivating enzyme transferring phosphate group to 3′-OH of the antibiotic [2]. Armed with this knowledge, he chemically derived 3′,4′-dideoxykanamycin, dibekacin, active against the resistant strains. This was a very early example of the rational design of antibiotics. It should also be noted that his success was supported by the results of basic research on the antibiotic-resistant bacteria. In fact, R-plasmids of the enteric bacteria were discovered in Japan in 1959 ahead of other countries.

Sarkomycin and mitomycin C, the latter of which is still being widely used in cancer chemotherapy, were discovered in the 1950s. This means that expansion of the targets of screening beyond antibiotics started early. In this direction Umezawa and his colleagues again showed leadership and creativity by initiating a new strategy of screening, i.e., screening of agents inhibiting enzymes involved in diseases or symptoms. Pepstatin, a specific inhibitor of pepsin and other aspartic proteases, is an initial example [3]. It is evident that his idea has opened up an aspect of the current rational approach of targeted screening.

Blasticidin S (1958) is the first antibiotic used in agriculture to prevent rice blast caused by a pathogenic fungus, *Piricuralia oryzae*. Use of its offspring such as kasugamycin [4] and polyoxin [5] has contributed to Japanese agriculture by reducing mercuric pesticides hitherto used in large amounts in fields. It may be appropriate to mention plant growth hormone giberellin briefly here in relation to these agro-antibiotics. It was originally found by Japanese scientists as a virulent agent of a plant pathogenic fungus, *Giberella fujikuroii*, which causes abnormal elongation of rice seedlings. The presence of an active substance in the culture filtrate of the fungus was reported very early in 1926 and the agent, giberellin, was identified by Yabuta and Sumiki in 1938 [6]. It is now produced on a large scale and used widely for producing seedless grapes in Japan.

2.3 Development of Applied Enzymology

Extensive screening of microbial strains proved to be a powerful tool for development of not only antibiotics but also industrial enzymes. Very early discoveries of several unique enzymes of great industrial usefulness and subsequent discoveries of a variety of unique applied enzymes of microbial origins conferred one of the characteristic features on the current biotechnology in Japan (Table 1).

In addition to dried kelp that provided monosodium L-glutamate, dried fish meat of skipjack tuna has been another traditional seasoning material for cooking in Japan. A preliminary paper describing inosinic acid as the essence of this flavor appeared in 1913, long before the establishment of nucleotide chemistry. Kuninaka [7] reexamined this work and revealed that 5′-inosinic and guanylic acids, but not the 2′- and 3′-nucleotides, possess not only a potent flavor themselves but also potent flavor-enhancing activity in the presence of monosodium glutamate. Since only venom nuclease was known to cleave RNA to 5′-nucleotides, they screened microorganisms for the activity and found nuclease P1 from a *Penicillium* strain [8]. Success of the enzymatic processes to produce the nucleotides from yeast RNA triggered the next challenge of nucleotide biosynthesis as described below.

Discovery of glucose isomerase is a contribution originated from Japan, leading to worldwide application in the sugar industry. In 1965, Sato and Tsumura [9] discovered the enzyme from *Streptomyces* strains, and the batch reactor system with the *Streptomyces* hyphae as a catalyst was developed soon afterwards. Industrial production of fructose + glucose syrup by combined use of glucose isomerase and glucoamylase started in 1971.

In 1967 Arima and his colleagues [10] found an aspartic protease with potent milk-clotting activity from a fungus *Rhizomucor pusillus*. It was the first successful microbial milk-clotting enzyme, which was required to meet the global shortage of calf chymosin for cheese production. Their invention was quickly followed by the development of a similar fungal enzyme from a closely related species *R. miehei*, and these fungal enzymes had replaced almost half of the world demands for milk-coagulants until the recent introduction of recombinant chymosin.

Combined use of microbial enzymes as biocatalysts with chemical synthesis has its origin in the steroid transformation developed in the USA in the early 1950s. Arima and his group [11] invented a unique microbial conversion process, in which the aliphatic side-chain of cholesterol was cleaved to produce a steroid core as a starting material for chemical synthesis of steroid hormones. Yamada et al. discovered the reverse reaction of the pyridoxal-containing L-amino acid lyases and applied them to synthesize L-tryptophan and L-DOPA [12] from pyruvate, ammonia and corresponding aromatic compounds. Since these early achievements, a variety of unique processes with newly screened microbial enzymes as biocatalysts have been invented.

Discovery of alkalophilic bacteria and their alkaline enzymes by Horikoshi in 1971 [13] was a direct demonstration of the microbial diversity. Since then,

Table 1. Examples of useful enzymes of microbial origins discovered since 1950

Enzymes and/or Products[a]	Origins of enzymes	References
Penicillin acylase		J Agr Chem Soc 23:411(1950)
Glucoamylase	*Bacillus amylosacchariticus*	Proc Japan Acad 27:352(1951)
Nuclease P1/5′-ribonucleotides[a]	*Penicillium citrinum*	see text
Lipase	*Rhizopus delemer*	J Gen Appl Microbiol 10:257(1964)
Glucose isomerase	*Streptomyces* sp	see text
Milk-clotting protease	*Rhizomucor pusillus*	see text
Cholesterol transformation	*Arthrobacter simplex*	see text
Tyrosine-phenol lyase/L-DOPA[a]	*Enterobacteriaceae*	see text
Serratiopeptidase	*Serratia* sp	Agric Biol Chem 34:310(1970)
Asparaginase	*E. coli*	Agric Biol Chem 35:743(1971)
Heat-stable lipase	*Humicola ranuginosa*	Agric Biol Chem 36:1913(1972)
CGTase/cyclodextrin[a]	Alkalophilic *Bacillus*	Agric Biol Chem 40:935(1976)
Cholesterol oxidase	*Brevibacterium sterolicum*	Agric Biol Chem 38:149(1974)
Caprolactam hydrolase/L-lysine[a]	*Cryptococcus* sp	Agric Biol Chem 41:1327(1977)
Hydantoinase/D-amino acid[a]	*Pseudomonas striata*	J Ferm Technol 56:484(1978)
Lysyl endopeptidase/h-insulin[a]	*Achromobacter lyticus*	Nature 280:412(1979)
Nitryl hydratase/acrylamide[a]	*Pseudomonas chloraphis*	see text
Alkaline endolytic cellulase	Alkalophilic *Bacillus*	J Bacteriol 158:503(1984)
Arabinonucleoside[a]	*Enterobacter aerogenes*	Agric Biol Chem 49:3239(1985)
Fungal peroxidase	*Arthromyces/Coprinus*	Agric Biol Chem 50:247(1986)
Cephalosporin acylase	*Pseudomonas* sp	J Bacteriol 169:5815(1987)
Protopectinase	*Bacillus subtilis*	Biosci Biotech Biochem 58:353(1994)
Lactone hydratase/D-pantolactone[a]	*Fusarium oxysporum*	see text
Trehalose[a]	*Arthrobacter* sp	see text
Transglutaminase	*Streptoverticillium* sp	see text
Proline hydroxylase	*Dactylosporandium* sp	see text

[a] Products produced by the enzyme processes are indicated instead of enzyme names.

a number of extracellular enzymes, such as proteases, amylases, cyclodextrin glucanotransferases (CGTase) and cellulases, with highly alkaline optimum pHs have been found mostly from alkalophilic *Bacillus* for various application. His work has initiated a trend leading to the current concept of extremophiles as described below.

It is also noted that Chibata and his colleagues [14] of Tanabe Pharmaceutical Co. started to use an immobilized enzyme for the optical resolution of DL-amino acids in 1969. The process included a fungal acylase immobilized on DEAE-Sephadex to hydrolyze *N*-acyl-L-amino acids selectively. This was the first industrial use of immobilized enzymes leading to the present concept of bioreactors.

2.4 New Vista Opened by Amino Acid Production

Discovery of glutamate production was a milestone in the history of Japanese process biotechnology, not only because of its own originality but also due to its role in creating a new paradigm of bioprocess technology leading to the current metabolic engineering. In 1956, Udaka and Kinoshita of Kyowa Fermentation Industry Co. reported the discovery of a novel bacterium *Corynebacterium glutamicum* (initially reported as *Micrococcus glutamicum*), which accumulated a large amount of L-glutamate from glucose and ammonia [15]. At that time this was almost an unpredictable phenomenon in the scope based upon the knowledge on the ethanol process. Technologically, a smart assay system to detect L-glutamate-producing colonies by using a glutamate-requiring bacterium as an indicator was a key to the success in this screening (Fig. 1). Enhanced leakage of L-glutamate due to biotin-deficiency of the producing organism was found to play a central role in the large accumulation, and penicillin-treatment was invented to assure the leakage in the biotin-rich industrial media. Ajinomoto quickly followed to protect its original market by using a similar organism, *Brevibacterium flavum*, and several other companies also engaged in this promising field of biotechnology. Although the competition caused some confusion in nomenclature of these producing strains, it has resulted in recognition of the *Coryne*-form bacteria as a unique phylogenetic group in bacterial systematics.

It is remarkable that accumulation of L-lysine in large amounts by an auxotrophic mutant of *C. glutamicum* was achieved within a year after the report of the glutamate process. The research group of Kyowa found that a homoserine-requiring mutant of *C. glutamicum* accumulated large amounts of L-lysine instead of L-glutamate [16]. Although the molecular mechanisms of neither the feedback regulation of amino acid biosynthesis nor the *lac*-operon induction in *E. coli* had yet been clarified at that time, this work suggested the presence of some regulatory networks as a key to switch biosynthetic pathways of amino acids. Detailed regulatory mechanisms were then revealed in *E. coli*, and the basic information facilitated to construct mutant strains accumulating various L-amino acids; these are described in another chapter of this volume. The rational approach used in these developments can be assumed to be a new field of fermentation, which is now called metabolic engineering.

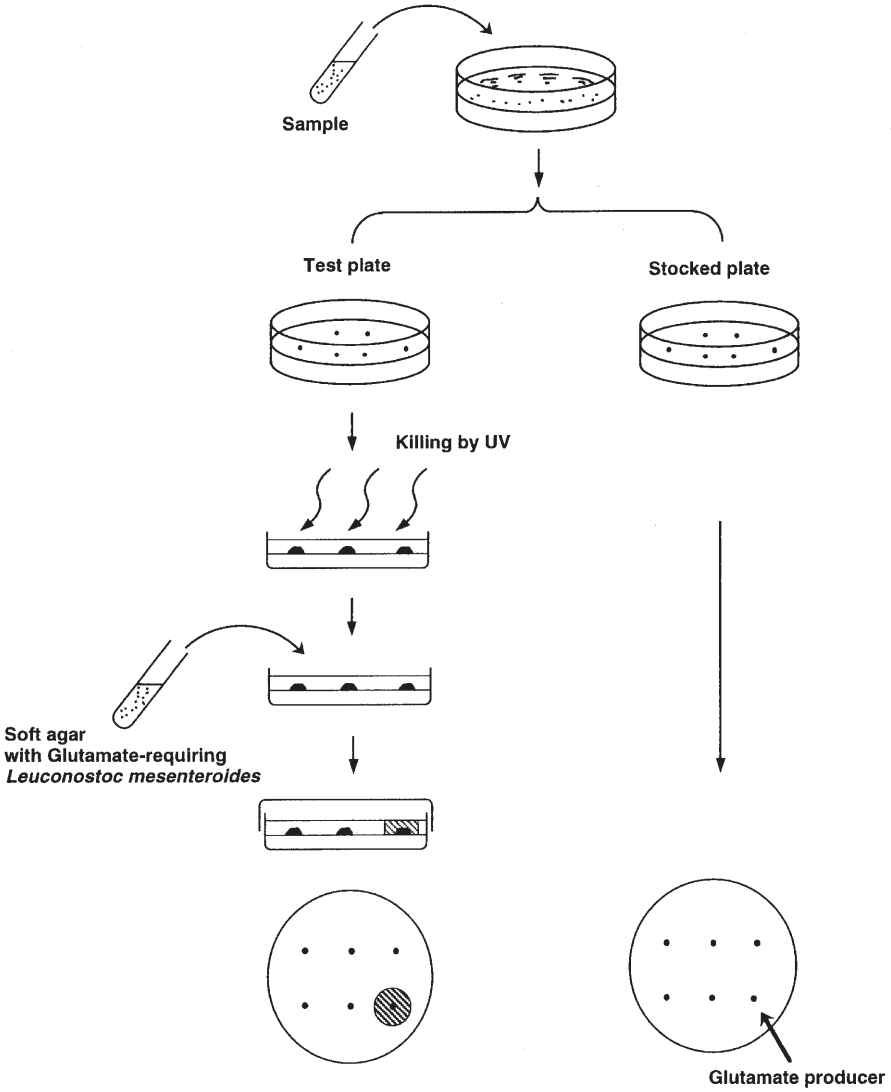


Fig. 1. Method of screening for glutamate producers

It is interesting to note that the discovery of marked flavor enhancing activity of 5′-inosinic and guanylic acids was made in 1960 just at the beginning of amino acid production [7]. Although the enzymatic hydrolysis of yeast RNA had achieved a distinct industrial success as described above, bioprocesses to produce these nucleotides were attempted by an approach similar to that used in developing the amino acid-producing strains. Accumulation of inosine and guanosine in large amounts was achieved by using adenine-requiring mutants of *Bacillus subtilis*, which were then chemically phosphoryalted to the corresponding

nucleotides [17]. On the other hand, Furuya et al. [18] reported direct accumulation of 5′-inosinic acid by an adenine-requiring mutant of *Brevibacterium ammoniagenes*, whose leakage seemed to be caused by the cell membrane abnormality induced at decreased concentrations of Mn^{2+}. A Mn^{2+}-insensitive mutant was derived from the strain so as to achieve the accumulation even in the presence of excessive Mn^{2+} in the industrial media [19]. These methods became a starting point for successive development of the nucleotide production systems as described below.

Several Japanese companies mainly conducted these innovative developments, and the severe technological race reproduced the stimulatory atmosphere of research and development that had once been observed at the beginning of the antibiotics industry. In such circumstance, the idea to manipulate genetically metabolic pathways was widely adopted in other bioprocesses as seen in construction of yeast strains with low diacetyl production for beer brewing [20]. In addition to creating metabolic engineering as a new paradigm of technology, these activities posed fundamental problems important in the basic microbiology. Enhanced leakage of L-glutamate in the *Coryne*-form bacteria is one such example, and elucidation of its molecular mechanisms is now a fascinating topic of the current bacterial physiology [21]. It should also be mentioned that experiences and techniques obtained during this research and development provided the basis for the following introduction of genetic engineering.

2.5 Beginning of Recombinant DNA Technology in Bioindustry

As soon as recombinant DNA technology appeared, many pharmaceutical and fermentation companies enthusiastically started research and development to produce heterologous proteins of human origin, mostly by using *E. coli* host-vector systems. Experience in microbial breeding and facilities of bioprocesses hitherto accumulated in Japanese industries enabled them to introduce some relevant licenses from abroad, while several cDNAs originally cloned in Japan, such as interferon-β [22], and IL-2 [23], were also developed for industrial production. Cloning and expression of chymosin cDNA in *E. coli* is noted as an early case applying this technology to targets other than medicinal use [24].

In order to apply recombinant DNA-technology to a wider variety of microorganisms, new host-vector systems were developed. Among them, the system for the amino acid-producing *Coryne*-form bacteria [25, 26] was useful for genetic analyses and molecular breeding of this group of bacteria. The system of *Bacillus brevis* is unique in its low proteolytic activity and high efficiency to secrete protein products, and was recently used for production of hEGF as described below [27].

Research and development of recombinant DNA technology has recently been expanding more and more rapidly. Global trends exemplified by the genome projects begin to exert profound effects on the future strategy of development, but those are beyond the scope of this brief review.

3
Recent Achievements of Applied Microbiology in Japan

3.1
Bioprocess Technology

The great success of amino acid and nucleotide processes revealed the capability of the genetic approach to overcome cognate regulatory networks in bacterial cells to achieve industrial production of metabolic intermediates of practical usefulness. Development of the host-vector system for the *Coryne*-form bacteria provided more freedom to manipulate the metabolic pathways. Since advances in the amino acid process are described in another chapter, here the recent development of the nucleotide production, especially unique hybrid processes constructed by coupling multiple microbial cells with different catalytic activities are described. Metabolic engineering for production of unsaturated fatty acids and a project to develop bacterial cellulose as a new industrial material are recent examples of research and development to expand the possibility of biotechnology. On the other hand, introduction of new technologies into the traditional brewery industry is producing several achievements such as recent molecular analyses of solid-state process of *Aspergillus oryzae*.

3.1.1
Metabolic Engineering for Production of Nucleotides

Bioprocesses to produce 5′-IMP and 5′-GMP have been classified into two types in general. One is a two-step process composed of production of nucleosides by bioprocess followed by chemical phosphorylation, and the another is the direct bioprocess accumulation of 5′-IMP and 5′-xanthilic acid (XMP). As the extension of the second one, the research group of Kyowa Fermentation Industry has developed the process to hybridize the strong ATP-regenerating activity of *Corynebacterium* with the reaction catalyzed by other microbial cells.

First they developed the process for production of 5′-GMP by hybridizing the XMP fermentation of *Corynebacterium ammoniagenes* with the energy-requiring amination reaction catalyzed by GMP synthase [28]:

$$5'\text{-XMP} + NH_3 + \text{ATP} \rightarrow 5'\text{-GMP} + \text{AMP} + \text{PPi}$$

In order to achieve the amination effectively, recombinant *E. coli* cells harboring the GMP synthase gene under the control of the λP_L promoter on a multi-copy plasmid was constructed, and the ATP-regeneration system in the *C. ammoniagenes* cells was used to supply ATP for this reaction. In order to assure the supply of ATP to the amination reaction, both of the two bacteria were treated with a mixture of detergent and solvent (polyoxyethylene stearylamine + xylene). The treatment made the cell membranes permeable to ATP but caused no damage to the ATP regeneration system in *C. ammoniagenes*. The whole process is operated in two steps: the first step is production of 5′-XMP by *C. ammoniagenes* alone, and then the recombinant *E. coli* cells are added to convert 5′-XMP to 5′-GMP

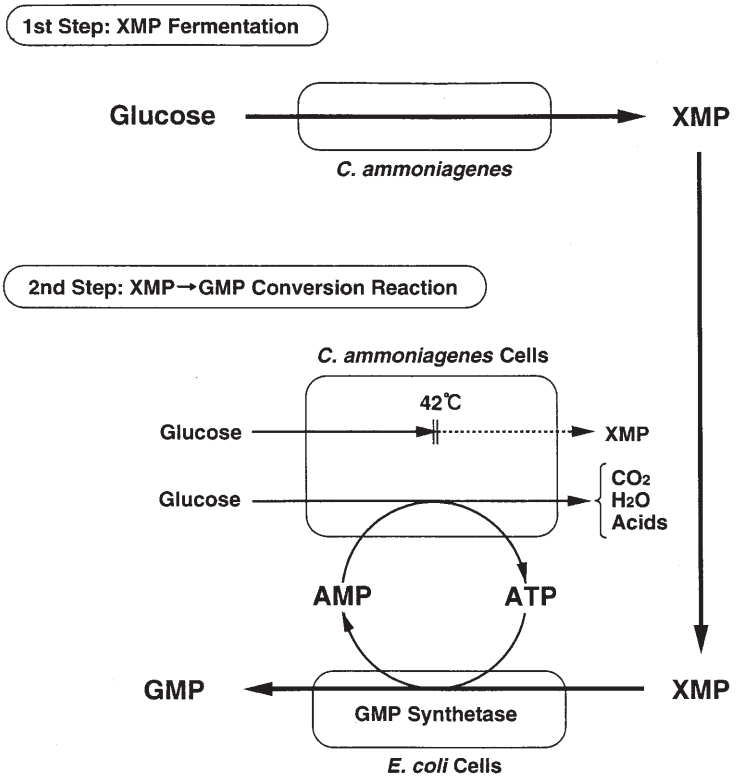


Fig. 2. Hybrid process to produce 5′-GMP catalyzed by cells of *Corynebacterium ammoniagenes* and recombinant *E. coli*

(Fig. 2). A final yield of 70 g l^{-1} as 5′-GMP · Na_2 · 7 H_2O with the conversion rate of 5′-XMP at 85% can be obtained.

A similar method was applied to produce 5′-IMP from inosine, which was accumulated by a mutant strain of *C. ammoniagenes* [29]. In order to convert inosine to 5′-IMP, recombinant *E. coli* with high activity of guanosine/inosine kinase was constructed by cloning the *E. coli* gene on an overexpressing plasmid. Although production of 5′-IMP was possible by the direct bioprocessing, the hybrid system allowed far higher bioprocess productivity. The process seems to have several advantages to the former processes in regards to not only productivity but also flexibility in applying to production a variety of phosphorylated compounds. Thus the system has been used for production of several phosphorylated materials such as CDP-choline [30], and UDP-galactose as well as globotriose [31].

3.1.2
Microbial Production of Polyunsaturated Fatty Acids

Polyunsaturated fatty acids (PUFAs) represented by linoleic and linolenic acids have been recognized as essential fatty acids for nutrition. Several members such as Mead, arachidonic, and eicosapentaenoic acids are known to be precursors of prostaglandins, thromboxanes, leucotrienes, etc., all of which have a variety of physiological activities like hormones. Some of them such as docosahexaenoic acid are recommended as dietary supplements for the prevention of heart diseases. Because of the increasing interest in and demand for the biologically important PUFAs, Shimizu and his group [32] extensively screened microorganisms that produced large amounts of PUFAs, and found a fungus *Mortiella alpina* and related species belonging to the genus *Mucorales*. Submerged cultivation of a strain of *M. alpina* for 5–7 days in the medium containing soybean oil yielded mycelia containing more than 50 wt% of arachidonic acid and the yield of arachidonic acid reached 4–8 kg l^{-1}. Thus the fungus is capable of being used as an effective resource of "Single Cell Oil" with high contents of arachidonic acid.

They further extended their work by isolating desaturase-deficient mutants of the fungus to manipulate biosynthetic pathways of PUFAs (Fig. 3). The main product of the strain, arachidonic acid, is synthesized via the ω-6 route, in which four kinds of desaturases Δ^5, Δ^6, Δ^9, and Δ^{12} are involved. Desaturase-deficient mutants have been isolated from several fungi and yeasts depending on the lethal or abnormal phenotype of development, but their mutations are limited to the Δ^9-desaturase. Shimizu and his group isolated mutants of *M. alpina* by analyzing fatty acids composition of all the colonies treated with nitrosoguanidine. This approach allowed them to obtain mutants of all the desaturases, which provides important information on their roles in the biosynthesis of PUFAs. Up to now, they have achieved production of dihomo-γ-linolenic acid by using a Δ^5-desaturase-deficient mutant, and of Mead acid by a Δ^{12}-desaturase-defective mutant.

3.1.3
Production of Bacterial Cellulose

It is well known that some strains of *Acetobacter*, namely *A. xylinum*, produce large amounts of cellulose as thick films in liquid stationary culture. Yamanaka et al. [33] noticed marked physical properties of dried bacterial cellulose sheets, which showed a high rigidity or Young's modulus similar to steel along with high internal loss to repress reverberation comparable to paper. His finding resulted in Sony's high quality acoustic transducer "Bio"diaphragm and thus stimulated interests in the possibility of this material. A research venture, Bio-Polymer Research Co., was organized by several companies with public budget supports for 1992–98 to exploit the possibility. In order to achieve mass production of bacterial cellulose at profitable costs, screening of hyper-producing strains in submerged culture and development of mechanical systems for the culture with extreme viscosity were carried out. As the consequence,

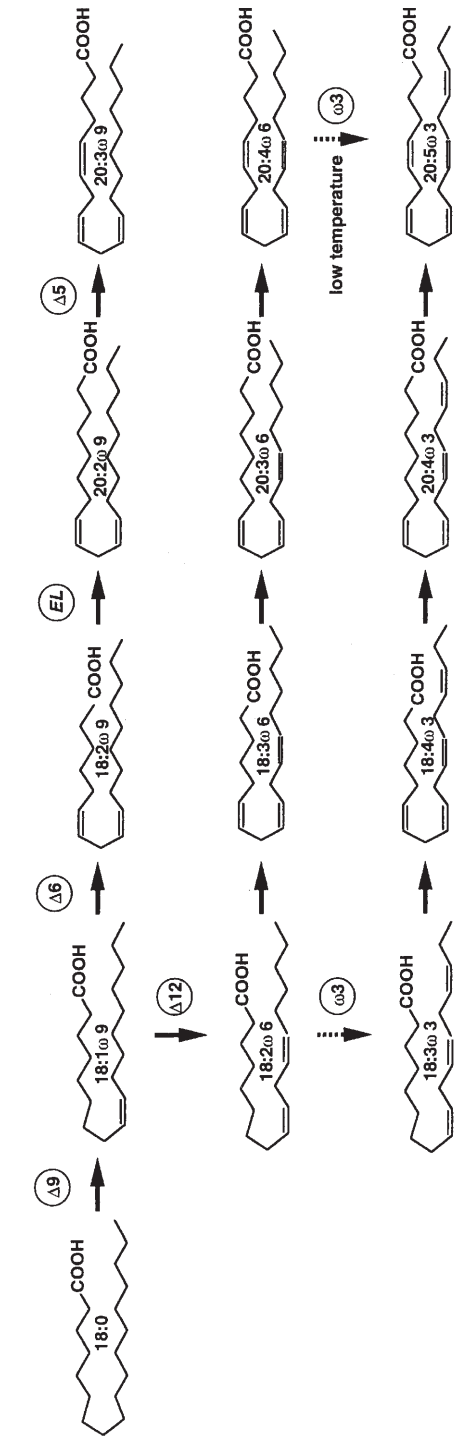


Fig. 3. Biosynthetic pathways of polyunsaturated fatty acids and the mutants of *Mortiella alpina*

yields exceeding 20 g l^{-1} cellulose in 40 h was achieved [34]. Although the productivity is still not sufficient to replace plant cellulose for normal purposes, the characteristic physico-chemical properties of bacterial cellulose will find its application in several industrial uses, an additive for paper making for example.

3.1.4
Molecular Biology of "Koji" for Sake Fermentation

In the sake brewing process *Aspergillus oryzae* is first grown on steamed rice as the solid-state culture called "koji", which is then immersed in water to start saccharification and fermentation with yeast. Recent application of recombinant DNA technology revealed a characteristic feature of gene expression in this solid-state culture. Hata et al. [35] found that a glucoamylase GlaB is expressed specifically in the solid-state cultures while another glucoamylase GlaA is expressed in the liquid cultures. The time-course of the *glaB* transcription in the solid-state cultures suggested that its expression is induced by low water-activity (*Aw*) and high temperatures. The results suggest that *glaB* belongs to the stress-induced genes. Fungal solid-state culture has several advantages, especially the capability of producing highly concentrated extracts of secreted enzymes. Further work will contribute to construct better fungal strains for traditional brewing processes and also for production of various extracellular proteins and other substances in solid-state cultures.

3.2
Application of Enzymes

Since many enzymes have capacities to catalyze reactions with even unnatural substrates and to produce unnatural compounds, hybrid use of enzymes as biocatalysts with chemical synthesis can realize processes to produce useful substances with higher flexibility than processes with growing cells. Discovery of novel microbial enzymes with required specificity by screening is a key to the establishment of such a hybrid processes. Many successful achievements in Japan are observed in this unique field of biotechnology. Application of nitrile hydratase to production of acrylonitriles has proved that biocatalysts can be applied to production of commodity chemicals beyond the presumed limitation of fine chemicals. Discovery of the enzymatic reactions to produce trehalose from starch is an example that reveals the possibility of microbial screening or what remains undiscovered in the microbial world. The importance of developing new application is also crucial in this field as shown in the case of transglutaminase and alkaline cellulase.

3.2.1
Amides Production by Nitrile Hydratases

Nitriles had long been assumed to be almost xenophilic for microbial metabolism. The group of Yamada, Kyoto University, and the research group of Nitto

Chemical Industry Co., demonstrated the presence of nitrile hydratase catalyzing conversion of aliphatic nitriles to corresponding amides [36]:

$$R \cdot CN + H_2O \rightarrow R \cdot CONH_2$$

They conducted joint development of the industrial process to produce acrylamide from acrylonitrile by the enzyme. Yamada and his colleagues also carried out extensive studies to reveal properties of the enzymes as well as screening of the enzyme from a variety of bacterial strains. These works have revealed the presence of several different types of nitrile hydratases, especially Co- and Fe-containing enzymes, in various bacteria. Among them, the Co-containing high-molecular-mass nitrile hydratase from *Rhodococcus rhodochrous* J1 showed the most effective productivity of acrylamide, and Nitto has constructed a plant equipped with a bioreactor using the immobilized bacterial cells as a biocatalyst, which contains highly elevated amounts of this enzyme exceeding 50% of total cellular proteins. The reactor produces acrylamide continuously at a concentration exceeding 50%. The enzymatic process surpasses the former Cu-catalyzed process in regard to several points such as the quality of the product with actual null contents of byproducts. This system is highly evaluated as the first successful case to apply biocatalysts for large-scale production of commodity chemicals.

The genes from both *Rhodococcus* and *Pseudomonas* strains were cloned and analyzed in cooperation with the group of Beppu, which revealed the tandem arrangement of the α- and β-subunits of the enzyme along with several auxiliary open reading frames including the amidase gene [37]. Kobayashi et al. [38] extended the genetic analyses to reveal the regulatory circuit, while Hashimoto et al. [39] developed a host-vector system of the *Rhodococcus* strain for effective expression of the genes. The Fe-containing nitrile hydratase had been known to show a curious property requiring irradiation with visible light for its activation. Recently the group of Endo et al. [40] has elucidated the mechanism on the basis of its fine three-dimensional structure. The most interesting feature is the presence of an NO molecule at the active center in the α-subunit, and the NO-binding stimulated by irradiation is the key to the photoactivation. The structural information will be useful for protein engineering of the Co-enzyme because of close homology in the sequences between these enzymes. Thus the time has come when the process is further improved by protein engineering.

Meanwhile, Yamada and his colleagues found that *R. rhodochrous* J1 contains another nitrile hydratase possessing different substrate specificity with preference for aromatic nitriles. By using this enzyme, they developed an industrial process to produce nicotinamide from 3-cyanopyridine (Fig. 4) [41]. The process

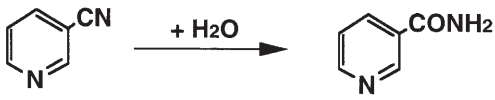


Fig. 4. Conversion of 3-cyanopyridine to nicotinamide by aromatic nitrilase

showed remarkable productivity converting 1.41 kg of 3-cyanopyridine suspended in 1 l of water to 1.4 kg of solidified nicotinamide crystals containing a small amount of residual water. The LONZA group has constructed a plant based on this process in China in 1997.

3.2.2
Optical Resolution of Pantolactone by Lactonehydrolase

D-Pantolactone, the γ-lactone of D-pantoic acid, is an important starting chiral material for the synthesis of a vitamin, D-pantothenic acid, which is mainly used as an additive for animal feeds and for various pharmaceutical products. Several derivatives of D-pantothenic acid, such as panthenyl alcohol, pantetheine, and coenzyme A, are also used as additives for infant formulae and as chemical reagents. The conventional synthesis of the vitamin involves optical resolution of racemic pantolactone by crystallization with an expensive alkaloid. Shimizu and his group [42] has developed several enzymatic processes to overcome this difficulty, and their recent achievement by using a specific lactonehydrolase is now being industrialized. Through extensive screening they observed that several microorganisms possessed the activity to hydrolyze aldonolactones with opposite stereospecifity. The enzyme selected from a fungus *Fusarium oxysporum* specifically hydrolyzes D-pantolactone to produce D-pantoic acid with optical purity of 96% e.e. In practice the hydrolysis is conducted by immobilized fungal cells entrapped into calcium alginate. The remaining L-pantolactone is easily recovered by extraction with solvent, and racemized for further recycling.

3.2.3
Proline Hydroxylase for Production of L-Hydroxyproline

Hydroxyproline is a useful chiral synthon for chemical synthesis of pharmaceuticals. It is also used as an additive for cosmetics due to its water-holding activity. Among the eight possible stereoisomers, only *trans*-4-hydroxy-L-proline (t-4HYP) is abundant in nature as a component of collagens in animal tissues, which is formed by post-translational hydroxylation by procollagen-proline dioxygenase. The research group of Kyowa Fermentation Industry Co. discovered a microbial enzyme catalyzing hydroxylation of free L-proline to t-4HYP, and developed an enzymatic process to produce t-4HYP by using a recombinant strain of *E. coli* [43].

First they developed a sensitive and hydroxyproline-specific detection method by HPLC to measure all stereoisomers of hydroxyproline at the picomole level. By using the method, more than 3000 actinomycete strains were screened for, and 8 strains were selected as the producers of proline 4-hydroxylase. The enzyme was purified from a strain belonging to *Dactylosporangium,* and the gene was cloned on the basis of its partial amino acid sequence. A recombinant *E. coli* strain carrying the gene on a high expression plasmid was constructed. Since the enzyme required 2-oxoglutamate to catalyze hydroxylation reaction, the conditions to assure regeneration of 2-oxoglutamate in the recombinant *E. coli* cells were established. Thus industrial production of t-4HYP from L-proline was established by using the recombinant *E. coli* cells as a biocatalyst.

3.2.4
Alkaline Cellulase as an Additive of Laundry Detergent

Since the extensive works of Horikoshi, various extracellular alkaline enzymes produced by alkalophilic bacteria have been developed for various applications. Kao Co. has initiated a unique application of alkaline cellulase as an additive of laundry detergent [44]. Although alkaline proteases have been widely used as the additive to remove proteinaceous materials in soiled clothes, it is not sufficient to remove soils in cotton fabrics, which is the major material used for clothes in Japan. The researchers of Kao found that an alkaline cellulase of *endo*-lytic type produced by an alkalophilic *Bacillus* strains was effective in removing soils from cotton fabrics without degradation or reduction of the tensile strength of the cotton fibers. They selected *Bacillus* sp. KSM-635 as the best strain, which produced the enzyme almost constitutively even in the absence of cellulosic substances. Hyperproducing strains were derived by successive mutagenesis and gene cloning. For example, they reported enhanced production of the enzyme by the mutants resistant to vancomycin and ristocetin. In 1987, Kao developed a new type of detergent, including the alkaline cellulase, which scored a big success in the market.

3.2.5
Transglutaminase to Modify Food Proteins

Transglutaminase catalyzes an acyl transfer reaction between the γ-carboxyamide group of peptide-bound glutamine residues and a variety of primary amines including the ε-amino group of lysine residues. The ε-(γ-glutamyl)lysine cross-linkings exist in proteins in the connective tissue and others and are involved in various physiological phenomena such as wound healing and epidermal keratinization. A similar enzyme was known to play an important role in the process to mold fish protein pastes into a Japanese popular foodstuff "kamaboko." A food research group of Ajinomoto Co. conducted the feasibility studies to confirm rapid gelation of several food protein solutions by the enzyme obtained from guinea-pig liver, which led to the following development of the microbial transglutaminase jointly with Amano Pharmaceutical Co. [45].

Microbial screening led to the discovery of a variant strain of *Streptoverticillium mobaraense* that produced a hitherto unknown microbial extracellular transglutaminase. The enzyme is capable of gelling concentrated solutions of proteins such as soybean proteins, milk proteins, and gelatin and myosin of various origins to produce gels with novel physical properties. An interesting application of the enzyme is production of restructured meat like steaks and fillets by binding meat pieces. The enzyme also causes crosslinking of two or more different proteins to produce new protein conjugates with novel functions. For instance, conjugation of milk casein or soya globulins to an egg glycoprotein, ovomucin, markedly increases the emulsifying activity of the parent proteins. It is possible to improve nutritive values of various food proteins by incorporating essential amino acids covalently. Due to these multiple functions, the enzyme is now finding a vast variety of applications in food processing.

3.2.6
Enzymatic Conversion of Starch to Trehalose

Trehalose (α-D-gucopyranosyl α-D-glucopyronoside) is a non-reducing sugar with sweet taste of good quality. The sugar is known as a stabilizer of proteins and a protector of the plant and animal tissues from damage by desiccation and freezing. Because of these characteristics, the sugar is expected to be an interesting new material for processed foods and medicinal and cosmetic uses, but its application has been limited due to the small supply, depending on extraction from yeast cells. Several attempts to use enzyme reactions such as reverse reactions of trehalase and trehalose phosphorylase had failed due to low productivity. The research group of Hayashibara Biochemical Laboratories, Inc. succeeded in finding a straightforward way to convert starch to trehalose by use of a microbial enzyme system [46, 47].

The activity was screened for by culturing all the isolated colonies in the medium containing maltopentaose as a permeable substrate and detecting trehalose by thin layer chromatography. A bacterial strain belonging to *Arthrobacter* was found to possess potent activity, which produced trehalose from dextrin or amylose by two unique enzymes, maltooligosyltrehalose synthase (MTSase) and maltooligosyltrehalose trehalohydrolase (MTHase) (Fig. 5). MTSase catalyzes intramolecular *trans*-glycosylation to convert a terminal α-1,4 glycosidic linkage of amylose to an α,α-1,1 linkage, while MTHase catalyzes selective hydrolysis of the intermediary product to release trehalose. Since the α-1,6 branching structure in starch inhibits the reaction, combined use of the debranching enzyme isoamylase is important to achieve high yields from starch. In 1995 Hayashibara started industrial production of trehalose by a process based on this enzyme system and the production is reportedly increasing rapidly.

Both MTSase and MTHase are localized within the cells and widely distributed among various bacterial species including Archaebacteria. Cloning and genetic analyses revealed that the genes encoding MTSase, MTHase, and isoamylase are located in a cluster in their genome [48]. These results imply that the enzyme system plays a role in synthesizing trehalose as an energy reservoir from starch in the cells of these bacteria.

3.3
Secondary Metabolites

Exploiting new physiologically active compounds beyond anti-microbial activities from microbial resources has now become the major trend. Pravastatin and FK506 discovered in Japan are the most successful examples. It is noted that in many cases microbial products obtained by screening are chemically or enzymatically modified to develop practically useful pharmaceuticals. In other words the role of microbial secondary metabolites as lead compounds for chemical synthesis is becoming clearer. Pharmaceuticals of microbial origins, which have been developed recently or are under development, are listed below and in Fig. 6. Many metabolites with interesting biological activities are omitted

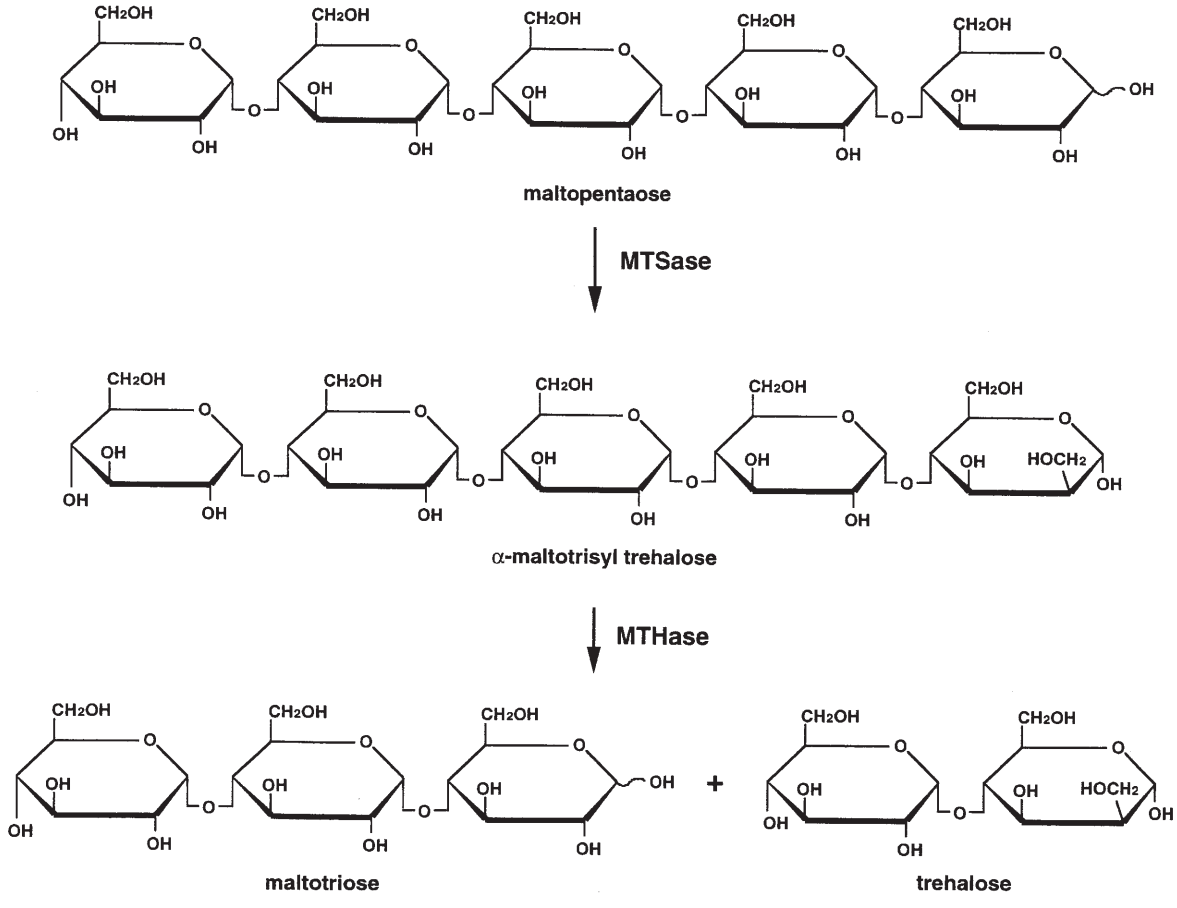
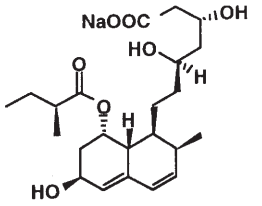


Fig. 5. Enzymatic system to produce trehalose from maltopentaose or amylose



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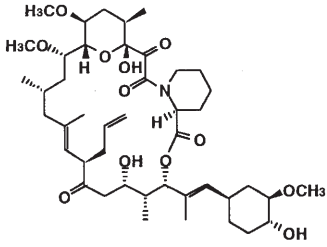

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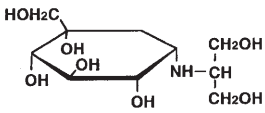

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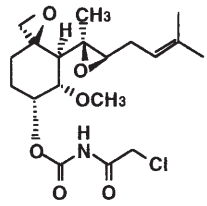

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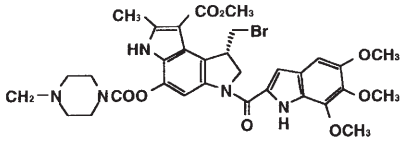

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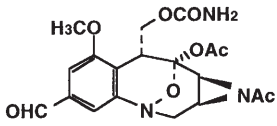

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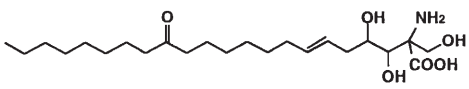

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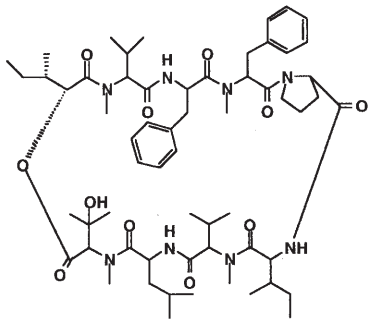

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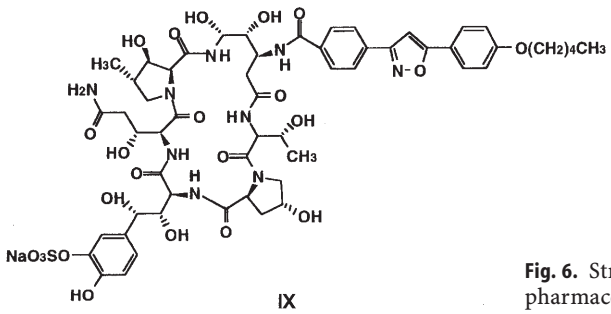


IX

Fig. 6. Structure of secondary metabolites of pharmaceutical use from microbial origins

because they are currently not successful as pharmaceuticals. It should be noted, however, they are useful in providing information about novel molecular targets for the future development.

Actinomycetes are the most important resources of these secondary metabolites. Recent advances of molecular genetics in this genus have enabled us to elucidate not only the organization of biosynthetic genes for their secondary metabolites but also regulatory mechanisms closely linked to the cellular differentiation processes. Although such information has so far not been successful in contributing to practical strain improvement, rational approach of combinatorial biosynthesis is expected to be useful in generating new compounds.

3.3.1 *Pharmaceuticals of Microbial Origins*

3.3.1.1 Pravastatin (I)

An elevated plasma cholesterol level has long been recognized as a major risk factor for atherosclerotic disease, specifically for coronary heart diseases. A group of microbial products with potent activity to inhibit 3-hydroxy-3-methylglutaryl-CoA reductase are now widely used for treating these patients. Mevastatin, the prototype of these agents, was discovered by Sankyo Co. from extracts of a *Penicillium citrium* strain in 1975 [49]. They screened for the activity by using an assay system in which inhibition by microbial products of incorporation of ^{14}C-labeled acetate into the sterol fraction was determined in rat liver tissues. Later Sankyo developed pravastatin possessing a hydroxyl group at the 6β position, which showed improved tissue-selective inhibition of cholesterol synthesis. Pravastatin is produced by microbial transformation of mevastatin catalyzed by cytochrome P-450 of *Streptomyces carbophilus* [50].

3.3.1.2 FK506 (Tacrolimus) (II)

Cyclosporin A, originally isolated as an anti-fungal antibiotic, was found to possess immunosuppressive activity and applied to suppress graft rejection in organ transplantation. Researchers of Fujisawa Pharmaceutical Co. constructed an in-vitro assay system to screen microorganisms for immunosuppressive agents by detecting the activity to inhibit the T-cell activation, and found FK506 produced by *Streptomyces tsukubaensis* [51]. It is now widely used in the world as an alternative agent, especially effective for transplant patients resistant to cyclosporin A. *cis-trans* Peptidyl-prolyl isomerase, FKBP, was identified as a binding protein of FK506. In addition, specific interaction of a calcium binding protein, calcineurin, with the complex of FKBP-FK506, was revealed to be a key to the T-cell signaling pathways to express the immuno-suppressive effect of FK506 [52].

3.3.1.3
Voglibose (III)

Voglibose is an *N*-substituted derivative of a pseudo-amino sugar developed by Takeda Pharmaceutical Co. as a potent α-glucosidase inhibitor for chemotherapy of diabetes [53]. It was derived from an antifungal antibiotic validamycin A produced by *Streptomyces hygroscopicus* var. *limoneus*, which was originally developed as a pesticide against sheath blight of rice plant caused by *Rhyzoctonia solani*. Validamycin A is first converted to a pseudo-amino sugar, valienamine, through microbial hydrolysis by *Flavobacterium saccharophilum*, and valienamine is then chemically converted to voglibose via valiolamine. This is a typical example of a microbial metabolite playing as a lead to develop new pharmaceutical agents.

3.3.1.4
Pharmaceuticals under Development

Fumagillin produced by *Aspergillus fumigatus* was found to inhibit angiogenesis. Takeda is developing TNP-470 (AGM-1470) (**IV**), a derivative of fumagillin, as an anti-tumor agent with a rather broad anti-cancer spectrum [54]. Recent identification of methionylaminopeptidase 2 as a molecular target of fumagillin and AGM-1470 provides a clue for future screening [55]. Two other anti-tumor agents with DNA-attacking activity, KW2189 (**V**) [56] derived from duocarmycin and FK317 (**VI**) derived from FR900482 [57], are being developed by Kyowa and Fujisawa, respectively.

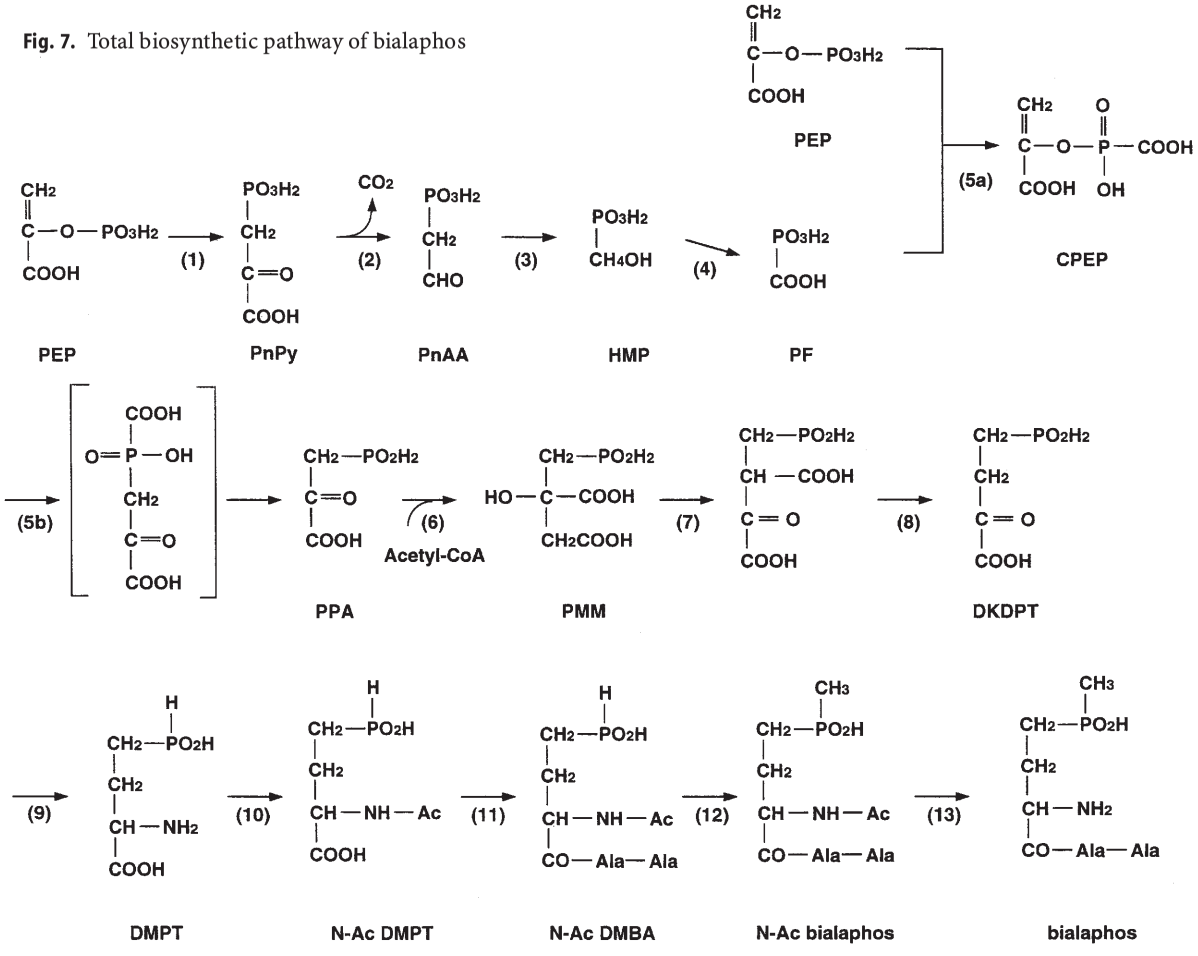
Fujita and his colleagues [58] discovered ISP-1 (**VII**) produced by the basidiomycete *Isaria sinclairii* as a potent immunosuppressive agent with a mechanism different from those of cyclosporin A and FK506. ISP-1 expresses its activity through induction of apoptosis of cytotoxic T-cells, which is probably due to inhibition of sphingosin biosynthesis [59]. Yoshitomi Pharmaceutical Co. is developing its derivative FTY720 for practical application.

FK463 (**VIII**) is a water-soluble lipopeptide derived from WF11899 A [60], which inhibits 1,3-β-glucan synthesis in the fungal cell wall. The agent is being developed by Fujisawa for deep sheeted mycoses. Aureobasidin A (**IX**), a cyclic peptide produced by the fungus *Aureobasidium pullulans*, was obtained according to its characteristic antifungal activity especially effective against *Candida* and *Cryptococcus* [61]. Although its development by Takara Brewery Co. seems to have been stopped, recent discovery that the activity is due to inhibition of inositolphosphoryl-ceramide synthase suggests a new molecular target for the future screening of antifungal agents [62].

3.3.2
Molecular Genetics of Secondary Metabolism

Bialaphos produced by *Streptomyces hygroscopicus* is a derivative of a unique C-P compound, phosphinotricin, and both are used as herbicides due to their activity to inhibit glutamine synthetase and thus to prevent detoxification of

Fig. 7. Total biosynthetic pathway of bialaphos



ammonia in plants. The research group of Meiji Seika Co. and Thompson [63] cooperated to analyze the biaplaphos synthetic gene cluster. They first isolated a number of mutants defective in the antibiotic synthesis and classified them according to their complementation patterns. Then they cloned the cluster of 23 kb and identified major biosynthetic genes by using the host-vector systems of the producing organism that they had developed. In combination of the genetic analyses, Seto [64] identified intermediates and enzymatic reactions directed by the gene products and finally clarified the total biosynthetic pathway (Fig. 7).

Dairi [65] investigated the application of recombinant DNA technology to the rare actinomycete *Micromonospora olivasterospora*, which produces fortimicin A, an aminoglycoside antibiotic characterized by its pseudo-disaccharide structure. After constructing the self-cloning system for this organism, they cloned almost the whole sequences containing at least 13 biosynthetic genes as well as a self-resistance gene and clarified the pathway in detail.

The basic information about the biosynthetic pathways will be useful, especially in generating new compounds through shuffling biosynthetic genes for different secondary metabolites belonging to the same group such as the polyketide antibiotics. Recently Ikeda and Omura [66] have revealed the total biosynthetic gene cluster of an anti-helminthic polyketide, avermectin, from *Streptomyces avermitilis*, and are exploiting the combinatorial approach.

During the course of elucidating the genetic instability of streptomycin production in *Streptomyces griseus*, Hara and Beppu [67] rediscovered A-factor with γ-butyrolactone structure, which had first been described by Khoklov et al. in 1967 as an auto-regulatory factor to induce both streptomycin production and sporulation in this organism. Recent genetic and biochemical analyses conducted by Horinouchi [68] have revealed the molecular mechanism of A-factor, which is analogous to the hormonal control systems in eukaryotes. Y. Yamada [69] also clarified a similar signaling system by virginia butanolides in *Streptomyces virginiae*. In addition, they have revealed that various γ-butyrolactone homologues are involved as the autoregulatory factors of secondary metabolism in streptomycetes in general. We may expect that such signaling substances will be useful to control the activities of these bacteria in their original habitat of soil.

3.4
Genetic Engineering for Production of Heterologous Proteins

Table 2 presents a list of the heterologous proteins of mostly pharmaceutical use being produced or under development in Japan by using recombinant DNA technology. The number of items apparently remains almost similar to that of 10 years ago. It does not mean there is low activity in research in this field. Instead, cloning of genes encoding components involved in cellular functions is now becoming an essential step to construct assay systems to screen for low molecular pharmaceuticals. Several topics are selected here to show diverse activities in this field.

Table 2. Recombinant proteins being produced or under development in Japan

Proteins	Hosts	Proteins	Hosts
Interferon α	*E. coli*	Tissue plasminogen activator	Animal cell
Interferon β	*E. coli*	Erythropoietin	Animal cell
Interferon*f* Á	*E. coli*	Calsitonin	*E. coli*
Feline interferon	Silkworm	Human atrial natriuretic peptide	*E. coli*
Human growth	*E. coli*		hormone
Somatomedine C	*E. coli*	Human serum albumin[a]	*Pichia pastoris*
Interleukin 2	*E.coli*	Tissue necrosis factor[a]	*E. coli*
G-CSF	Animal cell/ *E.coli*	Superoxide dismutase[a]	*E. coli*
Human epidermal growth factor	*Bacillus brevis*	Fibronectin fragment[a]	*E. coli*
Hepatitis B surface antigen	*S. cerevisiae*	Thrombopoietin[a]	*E. coli*

[a] These proteins are still not commercialized but their industrial production is being developed.

3.4.1 *Protein Engineering of G-CSF*

Human granulocyte-colony stimulating factor (G-CSF) is one of the protein factors involved in the differentiation of granulocytes, a major component of leucocytes playing vital roles such as protecting the body from bacterial infection. Recombinant G-CSF is produced by several companies as a means of rectifying a deficiency of leukocytes, caused as a side effect of chemical or radiation cancer therapy. Researchers of Kyowa Fermentation Co. improved properties of G-CSF by protein engineering. They generated more than 100 mutants of G-CSF by introducing deletion, substitution, and insertion at various sites in the molecules and examined their activities. They focused especially on the *N*-terminal sequence conserved in most of the hematopoietic growth factors, and finally obtained a mutant with the activity three times higher than the original as well as improved heat-stability and higher affinity to the receptor on neutrophils [70]. The mutant KW-2228 is being used for clinical treatment.

3.4.2 *Host-Vector System of Bacillus brevis*

The host-vector system of *B. brevis* has advantages of high secretion yields of the heterologous proteins as well as very low proteolytic activity of the host [27]. These properties are intrinsic in the bacterium, which was isolated as the strains for "protein fermentation" by Udaka [71], actually as an extension of Udaka's famous invention of the amino acid bioprocess. One of the selected strains,

B. brevis HPD31, accumulated an extracellular protein in an amount exceeding 30 g l^{-1}, which is a component of the outer surface layer of the *Bacillus* cells. He cloned the gene and took its promoter and secretion leader sequences to construct vector plasmids allowing expression and secretion of heterologous proteins. They also established characteristic transformation conditions of *B. brevis*, which included alkali-treatment to remove the surface protein layer and incubation of the cells with DNA in the presence of polyethylene glycol. The system shows the highest secretion efficiency among other bacterial hosts. The prokaryotic nature of the host may also be useful to avoid inappropriate glycosylation, which will occur during the secretion process in the eukaryotic hosts.

Essentially the same system was adopted by the Higeta Soysauce Co. for production of human epidermal growth factor (hEGF). Temporal dosage of hEGF to sheep was found to induce complete depilation and thus useful in removing wool from sheep. The industrial production has started for its practical application in Australia [72].

3.4.3
Production of Human Serum Albumin

Human serum albumin (HSA) is consisted of 585 amino acid residues without sugar chains and its content in serum exceeds 60% of the total serum proteins. Although plasma-derived HSA is widely used as a pharmaceutical to treat hypoalbuminemia, its supply is inadequate and a potential risk of contamination by blood-derived pathogens also exists. In order to overcome these problems, recombinant HSA may be the best solution. The Green Cross Co. now integrated to Yoshitomi Pharmaceutical Co. has been concerned about this subject for a long time. HSA has several characteristic features as a pharmaceutical which makes its production difficult. Since its unit price is low in comparison with other pharmaceutical proteins, very effective productivity in the bioprocess is required. In addition, very high clinical dosage of HSA requires extremely high purity of the final product. To fulfill these requisites, they chose a host-vector system of the methanol-utilizing yeast *Pichia pastoris*, which was originally developed by Philips Petroleum Co. [73]. The HSA cDNA placed downstream of the promoter of the methanol oxidase gene *mAOX2* is integrated into the host chromosome. The constructed transformant produces a large amount of HSA extracellularly by using methanol as a sole carbon source, and thus assures a low cost for production. The downstream process to produce recombinant HSA with the required purity and properties identical to the plasma-derived one has been established [74]. Reportedly a new plant for its large-scale production will be launched within a year.

3.4.4
Cloning of Thrombopoietin cDNA

In addition to various hematopoietic growth factors such as erythropoietin and G-CSF, a specific factor to stimulate platelet reproduction had long been assumed but remained to be confirmed until recently. The factor, thrombopoietin (TPO),

was identified by four groups in 1994 almost simultaneously, one of which, Kirin Brewery Co., succeeded in filing the patent. They developed a specific in vitro assay system to detect TPO according to enhanced incorporation of radioactive 5-hydroxytryptamine, which is the marker of megakaryocytes or the TPO-stimulated differentiation. As the source of TPO they used the plasma of γ-irradiated rats. After 1.5×10^8 times purification, they obtained a 19 kDa glycoprotein with TPO activity and finally cloned both the rat and human TPO cDNAs [75]. Since aglycosylated TPO produced in *E. coli* host showed lesser activity in vivo, the polyethyleneglycol-modified derivative is being examined for its clinical use. This is a recent example of the successful cloning of original genes of pharmaceutical activity in Japanese industry.

3.5 Exploiting Microbial Diversity

Although microbial diversity was seen a priori as the basis of success in screening projects, it has been scientifically recognized just recently. The existence of microorganisms in extreme environments, such as high temperatures, high salt concentrations, and at unusual levels of pH or oxygen, is clear evidence for the marked adaptability of microorganisms as the basis of their diversity. Systematic screening of extremophiles has now proved the marked potential of microorganisms beyond our imagination. In addition, recent ecological aspects on microbial flora including symbiotic systems are being incorporated into projects to explore microbial diversity.

3.5.1 *Extremophiles*

Two groups have so far isolated hyperthermophilic archaea able to grow at temperatures close to or over 100 °C from marine hyperthermal vents around the Japan islands. A group of Japan Marine Science and Technology Center isolated a strain named *Pyrococcus horikosii*, which can grow at 80 – 102 °C [76]. It was selected as a target of a governmental genome project and its whole sequence has been determined recently [77]. Imanaka and his group studied various heat-stable enzymes from strain KOD1 or *P. kodakaraensis,* and revealed their characteristic features. For example, they showed that the β-subunit of chaperonin from *Pyrococcus* expressed in *E. coli* helps to solubilize over-expressed heterologous proteins in the host cells [78].

Hydrophobic organic solvents are highly toxic to living cells in general and kill most microorganisms at low concentrations. Although some microorganisms including *Pseudomonas* and *Nocardia* can assimilate toluene for example, their tolerance against the solvent is less than 0.3 vol %. Inoue and Horikoshi [79] discovered a toluene-resistant bacterium from normal soil, which could grow in the presence of more than 50 % toluene or the presence of a second phase of toluene in the two-phase solvent-water system. The bacterium, identified as a variant of *Pseudomonas putida,* also showed marked tolerance against cyclohexane, xylene, styrene, and heptanol. Such organisms are expected

to be useful to develop bioreactor systems, in which organic solvents will be used for increasing supply of poorly water-soluble substrates or for extracting hydrophilic products from the reaction system.

Imanaka and his group discovered a novel bacterium HD-1 from a natural oil spill, which possesses an unusual activity to degrade petroleum anaerobically. The bacterium grew anaerobically in the presence of CO_2 and H_2 as a sole carbon source and an energy source, respectively, and the growth was markedly enhanced by the addition of aromatic or aliphatic hydrocarbons. Analyses of the cells showed that the bacterium fixed CO_2 and produced alkanes and alkenes with carbon numbers from 14 to 30 [80]. Although metabolic pathways have not yet been clarified, these observations suggest the involvement of still unknown microbial activities in the carbon recycling in the terrestrial environment.

3.5.2 *Microbial Consortia or Symbiotic Systems*

Microbiology and its application are based on pure cultures usually obtained by colony isolation techniques. However, most microorganisms in natural environments might express their activities in association with other strains or species through not only antibiosis but also symbiosis. Activities of the symbiotic microbial flora as observed in termites' intestine are now attracting attention as the target of future application [81]. The difficulty to deal with such microbial flora is that their members frequently fail to form colonies and thus unable to be obtained as pure cultures. A typical example was a symbiotic system consisting of a novel thermophile *Symbiobacterium thermophilum,* whose growth absolutely depended on the coexistence of a thermophilic *Bacillus* strain. PCR was effectively used to detect and quantify the growth and growth factor of this symbiotic bacterium [82]. This case indicates that recent technological progress in microbial ecology plays an important role in opening up a hitherto unrecognized part of microbial world. Although primary expectation of these novel isolates is the discovery of new enzymes, metabolites, and their genes for application, the results will also lead to reevaluation of microbial activities in natural environments and thus may present the next stages for the future biotechnology.

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Microbial Production of Amino Acids in Japan

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The microbial biotechnology of amino acids production which was developed and industrialized in Japan have been summarized. The amino acids include L-glutamic acid, L-lysine, L-threonine, L-aspartic acid, L-alanine, L-cysteine, L-dihydroxyphenylalanine, D-*p*-hydroxyphenyl-glycine, and hydroxy-L-proline.

Keywords. Microbial production, Amino acid, L-Glutamic acid, L-Lysine, L-Threonine, L-Aspartic acid, L-Alanine, L-Cysteine, L-Dihydroxyphenylalanine, D-*p*-Hydroxyphenyl-glycine, Hydroxy-L-proline

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1 Introduction

In Japan, people have used a kind of sea weed – 'kelp' – for a long time as a source of flavour. They extracted sea weed leaves with boiled water and used the extracts

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as a kind of soup for seasoning food. The tasty compound in the sea weed was identified as monosodium glutamate by Professor Kikunae Ikeda in 1908. And it was produced industrially from wheat, soybean, and other plant proteins after hydrolysis by concentrated hydrochloric acid, but the economics of this method was critical.

In 1957, Kinoshita et al. reported a bacterium isolated and identified as *Micrococcus glutamicus* (reidentified later as *Corynebacterium glutamicum*). It produced L-glutamic acid in a culture medium in appreciable amounts and microbial production of monosodium glutamate was started. Thereafter, many bacteria were identified as good glutamic acid producers and were used for monosodium glutamate production in Japanese industries. After the successful introduction of the technology, various methods were searched for and developed for microbial production of other amino acids. Today a whole array of amino acids are produced by microbial methods and used in the fields of medicine and food technology, and in the chemical industry. Estimated output and production data in Japan and elsewhere are summarized in Table 1 [1].

The microbial methods for the production of amino acids are classified as follows:

1. Methods employing wild strain bacteria (L-glutamic acid, L-alanine, L-valine production)
2. Methods employing mutants (L-lysine, L-threonine, L-arginine, L-citrulline, L-ornithine, L-homoserine, L-trypophan, L-phenylalanine, L-tyrosine, L-histidine, etc.)
3. Precursor addition methods (L-threonine, L-isoleucine, L-tryptophan, etc.)
4. Enzymatic method (L-aspartic acid, L-alanine, L-cysteine, L-dihydroxyphenylalanine, D-*p*-hydroxyphenyl-glycine, etc.)
5. Methods employing strains bred by gene-, protein-, and metabolic engineering or by combinations of these types of engineering (hydroxy-L-proline)

In this paper some representative examples of microbial production of amino acid will be summarized and discussed [2].

2 L-Glutamic Acid

Glutamic acid is produced by *Corynebacterium glutamicum* in the presence of high concentrations of sugar and ammonium ions, appropriate concentrations of minerals, and limited concentrations of biotin under aerobic conditions. The amount of L-glutamate accumulated in the medium is around 100 g/l in 2–3 days [2].

A large number of glutamic acid-producing bacteria were reported after the first report on *Corynebacterium glutamicum*, including *Brevibacterium flavum*, *Brevibacterium lactofermentum*, *Brevibacterium thiogenitalis*, and *Microbacterium ammoniaphilum*. The general characteristics of these strains were: gram-positive, non-sporulating, non-motile, coccal, or rod-like; all require biotin for growth. Today almost all of these strains are thought to belong to the genus *Corynebacterium*.

Table 1. Amino acid production in Japan and the world in 1996

Amino acid	Amount (tons/year)		Method			
	Japan	World	Bio-synthesis	Enzyme synthesis	Chemical synthesis	Extraction
Glycine	14,000	22,000			❍	
L-Alanine	250	500		❍	❍	
DL-Alanine	1500	1500			❍	
L-Aspertate	3000	7000		❍		
L-Aspergine	60	60		❍		❍
L-Arginine and analogs	1000	1200	❍			
L-Cysteine and analogs	900	1500		❍		❍
L-Glutamate	85,000	1,000,000	❍			
L-Glutamine	1200	1300	❍			
L-Histidine	400	400	❍			
L-Isoleucine	350	400	❍			
L-Leucine	350	500				❍
L-Lysine	500	250,000	❍			
L-Methionine	200	300			❍	
DL-Methionine	35,000	350,000			❍	
L-Phenylalanine	2500	8000			❍	
L-Proline	250	350	❍			❍
L-Serine	100	200	❍	❍		
L-Threonine	350	4000	❍			
L-Tryptophan	400	500	❍	❍		
L-Tyronsine	70	120				❍
L-Valine	400	500	❍		❍	
L-Dihydroxy-phenylalanine	150	250		❍		
D-Phenylglycine and analogs	3000	5000		❍	❍	

Estimated by Japan Amino Acid Association

The carbon source most commonly used as a starting material is glucose, which is obtained by enzymatic hydrolysis of starch from corn, potato, and cassava. Waste molasses is also used since it is inexpensive, but it contains large amounts of biotin which inhibits the microbial glutamate synthesis. So it is necessary to add some other effective compounds to the medium to facilitate glutamate accumulation.

Acetic acid and ethanol are also good carbon sources for glutamate production. Ethanol seems to be used after conversion to acetic acid in the cells of the

bacterium. Some hydrocarbons like *n*-paraffins are also assimilated as a carbon source and a glutamate process using *n*-paraffins was established in an earlier case. But nowadays these non-sugar carbon sources, including acetic acid and ethanol, are no longer used for economical reasons.

A high concentration of a source of nitrogen is necessary to produce glutamate and ammonium gas, its solution, an appropriate inorganic salt, or urea are used in actual production. Inorganic salts like potassium phosphate and ferric and manganese salts are also important. The pH of the medium is controlled at 7 – 8 by the addition of ammonia gas or solution, and the added ammonium ions are also used as the nitrogen source.

Coryneform bacteria generally show strong activity in sugar assimilation and glutamate dehydrogenase is the enzyme responsible for glutamate biosynthesis. Glucose incorporated in the cell is degraded through an EMP pathway and part of a TCA cycle, and 2-oxo-glutarate formed in the cycle is aminated to glutamate by the action of glutamate dehydrogenase.

Biotin is an important factor in regulating the growth of the bacterium and glutamic acid production. Its suboptimal addition is essential to produce good amounts of glutamic acid in the medium. To use a starting material such as waste molasses, which contains excess amounts of biotin, the addition of penicillin to the medium during growth was found to be effective. Several saturated fatty acids or their esters were also found to function similarly to penicillin with regard to the production of glutamic acid. A glycerol requiring mutant of *Corynebacterium alkanolyticum* was induced and the mutant produced glutamic acid in appreciable amount without the addition of penicillin and without the affection of biotin concentration.

The facts that these treatments, the biotin limitation, the addition of sublethal amount of detergents or penicillin, and induction of glycerol-requiring mutant are essential in the glutamate process suggest that the cell surface of the bacteria is damaged under such conditions, and consequently leaking of glutamate takes place. This leakage theory has been generally accepted for a long time, but recently another theory of excretion of glutamate has been published in which the presence of exporter protein of glutamate on the cell surface of the bacterium is suggested [3, 4].

2-Oxoglutarate dehydrogenase complex (ODHC), which catalyzes the conversion of 2-oxo-glutarate to succinyl-CoA as the first step of succinate synthesis in the TCA cycle, was reported to decrease in the cells of bacteria under the conditions of glutamate production. The enzyme activity was also recently confirmed to become very low in the presence of the detergent, limited amounts of biotin, or penicillin [5]. These results suggest that one of the main causes for glutamate overproduction is the decrease of the 2-oxoglutarate dehydrogenase activity, and the bacterial strain disrupting the enzyme gene produced as much glutamate as the wild type of bacteria which were under conditions of glutamate overproduction.

Furthermore, a novel gene *dtsR* was cloned which rescues the detergent sensitivity of a mutant derived from a glutamate-producing bacterium *Corynebacterium glutamicum* [6]. The authors found that this gene *dtsR* encodes a putative component of a biotin-containing enzyme complex and has something to do

with fatty acid metabolism. They reported that the disruption of this gene causes constitutive production of glutamate even in the presence of excess amounts of biotin and suggested that the overproduction of glutamate is caused by the imbalance of the coupling between fatty acid and glutamate synthesis [7]. Successively they showed that inducers of glutamate overproduction such as Tween 40 and limited amounts of biotin reduced the level of DtsR which then triggered overproduction by decreasing the activity of ODHC [8].

In new work, Kyowa Hakko Kogyo in Japan and Degussa in Germany almost completed the analysis of the genomic DNA nucleotide sequence of *Corynebacterium glutamicum*.

Monosodium L-glutamate is produced worldwide at levels of around one million tons by the microbial method. Two Japanese company, Ajinomoto and Kyowa Hakko Kogyo, built factories and produced it in other countries, mainly in south east Asian areas. China, Korea, and Taiwan also produce large amounts of L-glutamate monosodium salt nowadays. This is used in the food industry as a seasoning to improve taste, its ester is used as a detergent, and the polymer as an artificial skin.

3 L-Lysine

L-Lysine is produced by some mutants induced from wild strain of glutamate-producing bacteria including *Corynebacterium glutamicum*, *Brevibacterium lactofermentum*, and *B. flavum* in the presence of high concentrations of sugar and ammonium ions at neutral pH and under aerobic condition [2].

The pathway of biosynthesis of L-lysine and L-threonine in *Corynebacterium glutamicum* is shown in Fig. 1. The first step, the formation of phosphoaspartate from aspartate, is catalyzed by aspertokinase and this enzyme is susceptible to the concerted feedback inhibition by L-lysine and L-threonine. The auxotrophic mutant of homoserine (or threonine plus methionine), lacking homoserine dehydrogenase, was constructed and found to produce L-lysine in the culture medium. Second, the mutants which show the threonine or methionine sensitive phenotype caused by the mutation on homoserine dehydrogenase (low activity) was also found to produce appreciable amounts of L-lysine in the culture medium. Furthermore, a lysine analogue (*S*-aminoethylcysteine) resistant mutant was obtained as an L-lysine producer and in this strain aspartokinase was insensitive to the feedback inhibition.

These characteristics of lysine producers are combined to produce much stronger lysine producing strains. In addition to these fundamental properties, further addition of leucine requiring mutation is effective to increase the amount of lysine since in the mutant dihydrodipycolinate synthase is released from repression by leucine. The precursors of lysine synthesis include phosphoenol-pyruvate, pyruvate, and acetylCoA. In addition, many mutations are induced in the lysine producers to supply sufficient amounts of these precursors in good balance. These are deletion mutants of pyruvate kinase and show low activity of pyruvate dehydrogenase, etc. Furthermore, an alanine requirement was also reported to be effective in increasing the lysine amount.

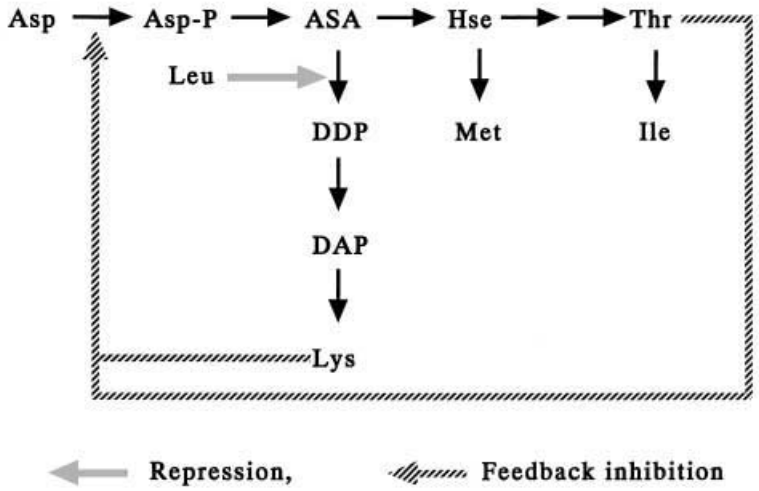

Fig. 1. Regulation of lysine biosynthesis. ASA, aspartate-β-semialdehyde; DDP, dihydrodipicolinate; DAP, α, ε-diaminopimelate; Hse, homoserine

Now the genes of the enzymes responsible for the biosynthesis of lysine in *Corynebacterium* have been cloned and the nucleotide sequences determined. They were the genes of aspartokinase, aspartate semialdehyde dehydrogenase, dihydrodipycolinate synthase, dihydrodipycolinate reductase, tetrahydrodipicolinate succinylase, succinyl diaminopimalate desuccinylase, diaminopimelate dehydrogenase, and diaminopimelate decarboxylase. The host-vector system of *Corynebacterium* was already established and the introduction of some genes which encoded the enzymes responsible for lysine biosynthesis was found to be effective in increasing the amounts of lysine produced. Those genes are those of aspartokinase and dihydrodipicolinate synthase.

A new gene *ldc* which encodes lysine decarboxylase was found in addition to the formerly known *cadA* in *Escherichia coli* and the enzyme purified from the overexpression strain. The lysine decarboxylase encoded by *ldc* is constitutively produced by *E. coli* cells though the *cadA* encodes an inducible one [9]. It is interesting to know of the existence of this new lysine decarboxylase in lysine-producing *Corynebacterium* and to investigate the effects of the deletion of the gene on the amounts of L-lysine production.

Vrljic et al. cloned a new gene *lysE* from *Corynebacterium glutamicum* and showed that it encodes the translocator which specifically exports L-lysin out of the cell [10]. Recently they analyzed the membrane topology of the gene product and showed that it is a member of a family of proteins found in some bacteria – *Escherichia coli*, *Bacillus subtilis*, *Mycobacterium tuberculosis*, and *Helicobacter pylori*. The authors suggested that LtsE superfamily members will prove to catalyze the export of a variety of biologically important solutes including amino acids [11 – 13].

Lysine is useful as a feed additive for swine and poultry, since their feeds such as grain and defatted soybea1ns contain lower amounts of lysine, which is one of

the essential amino acids for those livestocks. The estimated amount of L-lysine produced in the world is around 400,000 tons and almost all of this is supplied by Ajinomoto, Kyowa Hakko Kogyou, ADM, and BASF, who have built factories all over the world.

4 L-Threonine

L-Threonine is produced by some auxotrophic mutants and/or threonine-analog resistant mutants and those bred by gene engineering techniques. The bacteria are *Escherichia coli*, *Corynebacterium glutamicum*, *Brevibacterium lactofermentum*, *B. flavum, Serratia marcescens*, and *Proteus retgerii*.

The auxotrophic mutants of L-lysine, diaminopimelate, or L-methionine were found to produce L-threonine in the culture medium but the amount is not enough for practical production. A mutant resistant to an L-threonine analogue, α-amino-β-hydroxyvaleic acid (AHV), was obtained as an L-threonine producer and in this strain homoserine dehydrogenase was insensitive to feedback inhibition by L-threonine (see Fig. 1). The much stronger L-threonine-producing strains were obtained by the combination of auxotrophic mutations and AHV-resistant mutation. L-Threonine-producing mutant of *S. marcescens* was induced by the techniques of phage transduction. The strain has the following properties: deficiency of L-threonine-degrading enzymes, mutation in aspartokinase and homoserine dehydrogenase to be insensitive to feedback inhibition by L-threonine, mutation in L-threonine biosynthetic enzymes to release them from repression by L-threonine, mutation in aspartokinase to be insensitive to feedback inhibition by L-lysine, and mutation in aspartokinase and homoserine dehydrogenase to be released from the repression by L-methionine.

Recombinant DNA techniques were employed to improve the L-threonine producer. A threonine-deficient mutant of *E. coli* was transformed by the genes of threonine operon obtained from α-amino-β-hydroxyvaleric acid (AHV)-resistant and feedback-insensitive mutants to amplify the expression of enzymes and to increase the amount of L-threonine. *E. coli* mutant strain was also constructed to have amplified genes of threonine operon obtained from AHV-resistant and feedback-insensitive mutant by the action of Mu phage on the chromosomal DNA. This strain is used in France in the practical production of L-threonine. The productivity of bacterial strains developed as the L-threonine producer is summarized in Table 2 [14]. L-Threonine hyperproducing *E. coli* mutant, which can produce 100 g/l of L-threonine in 77 h, was constructed by Okamoto et al. who suggested that the strain has some impairment in L-threonine uptake function [15].

L-Threonine production by microbes was started in the 1970s, the auxotrophic and analog resistant mutant strains obtained for the purpose being cultured in the presence of amino acids which are required by the mutant.

L-Threonine is an essential amino acid for humans and some livestock animals including pigs and poultry. It is used as an additive in animal feed, medical products, food, and cosmetics. The amount of production is around 13,000 – 14,000 tons per year worldwide.

Table 2. Productivity of L-threonine by bacterial mutant strains

Strain	Culture time (h)	Produced amount (g/l)	Production rate (g/1/h)	First author
C. glutamicum	90	52	0.58	Katsumata
B. lactofermentum	100	58	0.58	Ishida
S. marcescens	96	100	1.04	Masuda
E. coli	72	65	0.90	Shimizu
E. coli	77	100	1.30	Okamoto

5 L-Aspartic Acid

L-Aspartate is produced by a one-step enzymatic method from fumarate and ammonia and by a two-step method from maleate via fumarate. The conversion of fumarate to L-aspartate is catalyzed by aspartase and maleate to fumarate by maleate isomerase:

$$\text{Maleate} \xrightarrow{\text{maleate isomerase}} \text{fumarate} \xrightarrow{\text{aspartase}} \text{L-asparatate}$$

The industrial L-aspartate production by enzymatic process was started in 1960 with a batchwise system using *E. coli* cells with high aspartase activity. At the beginning of 1973, aspartase extracted from *E. coli* cells were immobilized on ion exchange resin and L-aspartate was produced in a continuous reaction system using a column of the immobilized enzyme by Chibata and collaborators in Tanabe Seiyaku Co. Another system was started in 1973 – in which the cells of *E. coli* were immobilized by trapping in acrylamide gel lattice – and used in industrial production by Tanabe Seiyaku Co. In 1978, this trapping matrix changed to κ-carageenan, a polysaccharide obtained from seaweed. The productivity of L-aspartate was improved very much by this method and the yield became 100 tons/months using a 1-m^3 bioreactor [2]. In USA, immobilization of *E. coli* cells with high aspartase activity on polyurethane and polyazetidine were reported and the latter has shown the high activity of aspartase of 55.9 mol/h/kg cell wet weight [16].

A new system for the enzymatic production of L-aspartate was proposed and started in the 1990s. In this system, resting intact cells of coryneform bacteria were used without immobilization and with an ultrafiltration membrane. This bacterial strain possesses high maleate isomerase and aspartase activities thorough transformation of their genes. The plasmids introduced were stabilized and the cells were reused many times without any loss of activity and lysis [17].

L-Aspartate is used in parenteral nutrition and food additives, and as a starting material for the low-calorie sweetener aspartame, aspartyl-phenylalanine methyl ester. Recently, the possibility of using L-aspartate as a raw material for polymer production was studied very hard since it has three reactive residues in the molecule and the resulted polymers could be biodegradative. It is used as a detergent and chelating or water treating agent.

6
L-Alanine

L-Alanine is produced from L-aspartate by a one-step enzymatic method using aspartate β-decarboxylase:

$$\text{L-aspartate} \xrightarrow{\text{aspartate } \beta\text{-decarboxylase}} \text{L-alanine} + CO_2$$

Pseudomonas dacunhae was isolated, identified, and chosen as the most favorable strain for the production of L-alanine since it showed the highest activity with aspartate β-decarboxylase. At first, the production of L-alanine by immobilized cells were accomplished by *P. dacunhae* immobilized with polyacrilamide in Tanabe Seiyaku Co. The cells of *P. dacunhae* were immobilized with κ-carageenan, a polysaccharide obtained from a seaweed which has a good entrapping matrix properties. The column packed with the immobilized cells were used as a reactor for the continuous production of L-alanine. A closed column reactor was designed and used for the continuous production of L-alanine. In this column the enzyme reaction proceeded under high pressure, preventing the evolution of carbon dioxide gas. This column system is connected in tandem to an L-aspartate producing column system to produce L-alanine directly from fumarate. However, in this system, a side reaction caused by fumarase and alanine racemase in both bacteria *E. coli* and *P. dacunhae* reduced the yield significantly. The enzymes were inactivated by the treatment of both bacterial cells separately at high temperature and low pH [18]. Subsequent immobilization of these two kinds of bacterial cells in a κ-carageenan matrix allowed production of L-alanine in a single reactor without the production of the side products, malate and D-alanine:

$$\begin{array}{ccccc} \text{Fumarate} + NH_3 & \xrightarrow{\text{aspartase}} & \text{L-asparate} & \xrightarrow{\text{aspartate } \beta\text{-decarboxylase}} & \text{L-alanine} + CO_2 \\ \downarrow & & & & \downarrow \\ \text{malate} & & & & \text{D-alanine} \end{array}$$

L-Alanine is produced at a level of 10 tons/month using this kind of high pressure column reactor system. L-Alanine is useful as an additive to both entheral and parenteral nutrition, being a food additive with a sweet taste and bacteriostatic properties [2].

7
L-Cysteine

L-Cysteine had been produced by extraction from hair after hydrolysis with strong acid. However, this process has many problems such as too high energy costs, occurrence of bad smell, production of much acidic waste, and an unreliable supply of hair. In the 1970s a three-step enzymatic method was established by Ajinomoto Co. to produce L-cysteine from DL-2-amino-Δ^2-thiazoline-4-carboxylate(DL-ATC), a starting material of the chemical synthesis of L-cysteine. The enzymes catalyzing this process are DL-ATC racemase L-ATC hydrolase and *S*-carabamoyl-L-cysteine (SCC) hydrolase:

$$\text{L-ATC} \xrightarrow{\text{L-ATC hydrolase}} \text{S-carabamoyl-L-cysteine} \xrightarrow{\text{SCC hydrolase}} \text{L-cysteine}$$
$$\uparrow \text{ ATC racemase}$$
$$\text{D-ATC}$$

A bacterial strain isolated from soil and designated as *Pseudomonas thiazolinophilum* had shown the highest activity in producing L-cysteine from DL-ATC. The enzymes responsible for the conversion are inducible and the addition of DL-ATC to the culture medium is essential for high enzyme activities. Addition of Mn^{2+} and Fe^{2+} to the medium also contributed to increasing enzyme activity. The reaction proceeds by the addition of cells having high activity with the enzymes to the reaction mixture containing DL-ATC. Addition of hydroxylamine, an inhibitor of vitamin B6-dependent enzymes, to the reaction mixture is effective in preventing the degradation of the L-cysteine produced. Hydroxylamine inhibits an L-cysteine degrading enzyme, cysteine desulfhydrase. A mutant of this enzyme lacking was also obtained and used for the industrial production for L-cysteine. L-Cysteine produced in the reaction mixture is oxidized to L-cystine by aeration during reaction and precipitated as crystals. The amount of L-cysteine obtained from 40 g/l DL-ATC was 31.4 g/l, a 95% yield in molar ratio. This enzymatic production was started in 1982 by Ajinomoto Co.

S-Carboxymethyl-L-cysteine is also produced by the same enzymatic method with the corresponding starting material.

L-Cysteine is useful as a chemical, hair treatment agent, and food additive.

8 L-DOPA

L-DOPA is produced from pyrocatechol, pyruvate, and ammonia by a one-step enzyme reaction using tyrosine phenol-lyase:

$$\text{Pyrocathechol + pyruvate + ammonia} \xrightarrow{\text{tyrosine phenol-lyase}} \text{L-DOPA}$$

Tyrosine phenol-lyase (TPL) is a pyridoxal 5′-phosphate-dependent multifunctional enzyme and catalyzes degradation of tyrosine into phenol, pyruvate, and ammonia. This reaction is reversible and the reverse reaction is available to produce L-DOPA using pyrocatechol instead of phenol.

Erwinia herbicola was selected as the most favorable strain for L-DOPA production out of 1041 microbial strains tested. Culture conditions for the preparation of cells containing high TPL activity and reaction conditions for the synthesis of L-DOPA were optimized with this bacterium. Cells were cultivated at 28 °C for 28 h in a basal medium consisting 0.2% L-tyrosine, 0.2% KH_2PO_4, and 0.1% $MgSO_4 \cdot 7H_2O$ (pH 7.5). Various amounts of the nutrients were added to the basal medium. Additions of yeast extract, meat extract, polypeptone, and the hydrolyzate of soybean protein to the basal medium enhanced cell growth as well as the formation of TPL. Catabolite repression of biosynthesis of TPL was observed on adding glucose, pyruvate, and α-ketoglutarate to the medium at high concentrations. Glycerol was a suitable carbon source for cell growth as well as for the accumulation of the enzyme in growing cells. A marked increase

in enzyme formation was observed when glycerol was added together with succinate, fumarate, or malate. TPL is an inducible enzyme and the addition of L-tyrosine to the medium is essential for formation of the enzyme. L-Phenylalanine is not an inducer of TPL biosynthesis but works as a synergistic agent to induction by L-tyrosine. The activity of TPL increase five times by the addition of L-phenylalanine together with L-tyrosine in the medium. Cells of *E. herbicola* with high TPL activity were prepared by growing them at 28 °C for 28 h in a medium containing 0.2% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 2 ppm Fe^{2+} ($FeSO_4 \cdot 7H_2O$), 0.01% pyridoxine · HCl, 0.6% glycerol, 0.5% succinic acid, 0.1% DL-methionine, 0.2% DL-alanine, 0.05% glycine, 0.1% L-phenylalanine, and 12 ml of hydrolyzed soybean protein in 100 ml of tap water, with the pH controlled at 7.5 throughout cultivation. Under these conditions, TPL was efficiently accumulated in the cells of *E. herbicola* and made up about 10% of the total soluble cellular protein [19].

The enzymatic synthesis reaction of L-DOPA is carried out in a batchwise system with cells of *E. herbicola* with high TPL activity. Since pyruvate, one of the substrates, was unstable in the reaction mixture at a high temperature, low temperature was favored for the synthesis of L-DOPA. The reaction was carried out at 16 °C for 48 h in a reaction mixture containing various amounts of sodium pyruvate, 5 g of ammonium acetate, 0.6 g of pyrocatechol, 0.2 g of sodium sulfite, 0.1 g of EDTA, and cells harvested from 100 ml of broth, in a total volume of 100 ml. The pH was adjusted to 8.0 by the addition of ammonia. At 2-h intervals, sodium pyruvate and pyrocatechol were added to the reaction mixture to maintain the initial concentrations. The maximum synthesis of L-DOPA was obtained when the concentration of sodium pyruvate was kept at 0.5%. The substrates, pyrocatechol and pyruvate, were added at intervals to prevent the denaturation of TPL and to prevent byproduct formation. The addition of sodium sulfite is effective in maintaining the reactor in a reductive state and in preventing the oxidation of the L-DOPA produced. L-DOPA is insoluble in the reaction medium, so it appears as a crystalline precipitate during the reaction, at final amounts reaching 110 g/l [19–21].

Induction and repression mechanism of TPL of *E. herbicola* were studied and it was found that TPL biosynthesis is regulated on the transcriptional level. mRNA of TPL was increased by the addition of tyrosine and decreased by the addition of glucose to the medium. TyrR box, an operator-like region was found in the 5′ flanking region of its gene, *tpl*. TyrR box is a typical binding site of DNA where a regulator protein TyrR binds and regulates transcription of the regulon of enzymes or transporters responsible for aromatic amino acids biosyntheses or transport through cell membranes [22–24].

L-DOPA is the precursor of the neurotransmitter dopamine and useful as a treatment for Parkinsonism, in which the amount of dopamine in the brain of the patient is insufficient. Worldwide L-DOPA production is around 250 tons/year. It had been mainly produced by a chemical synthetic method that involved eight chemical unit reaction steps including optical resolution (Table 3). The enzymatic L-DOPA production method via *Erwinia* TPL is a simple one-step method and one of the most economic processes to date. It was first used in 1993 by Ajinomoto Co.

Table 3. Comparison of enzymatic and chemical L-DOPA production methods

	Enzymatic method	Chemical method
Starting materials	Pyrocatechol, Pyruvate, Ammonia	Vanillin, hydantoin, acetic anhydride, hydrogen
Total number of unit reaction	1	8
Optical resolution	Unnecessary	Necessary
Equipment	Commonly usable fermenter	Specific plant
Period (days)	3	15
By-products	H_2O	Ammonia, CO_2, acetate

9 D-*p*-Hydroxyphenylglycine

D-*p*-Hydroxyphenylglycine (D-HPG) is a useful starting material for the production of semisynthetic penicillins and cephalosporins, such as amoxicillin and cephadoxel. D-HPG is produced from DL-*p*-hydroxyphenylhydantoin (DL-HPH) by a two-step enzymatic method.

The starting material DL-HPH is synthesized by the amidoalkylation of phenol. DL-HPH is completely hydrolyzed to *N*-carbamoyl-D-*p*-HPG by microbial hydantoinase. This *N*-carbamoyl-D-*p*-HPG is then hydrolyzed to D-HPG by microbial *N*-carbamoyl-D-*p*-HPG hydrolase:

$$\begin{array}{ccc} \text{D-}p\text{-OH-phenylhydantoin} & \xrightarrow{\text{hydantoinase}} & \text{N-carbamoyl-D-}p\text{-HPG} \\ \uparrow \text{ spontaneous racemization} & & \downarrow \text{ D-carbamoylase} \\ \text{L-}p\text{-OH-phenylhydantoin} & & \text{D-}p\text{-HPG} \end{array}$$

DL-HPH is spontaneously easily racemized at the slightly alkaline pH but not with *N*-carbamoyl-D-*p*-HPG. Then, during the reaction, only D-HPH is hydrolyzed by hydantoinase to form D-HPG via *N*-carbamoyl-D-*p*-HPG. L-HPH is racemized and hydrolyzed by hydantoinase to form D-HPG. Finally DL-HPH in the reaction mixture is completely hydrolyzed to D-HPG.

A high D-hydantoin hydrolase activity was found in some bacteria belonging to the genera *Bacillus*, *Pseudomonas*, *Aerobacter*, *Agrobacterium*, and *Corynebacterium* and in actinomycetes belonging to the genera *Streptomyces* and *Actinoplanes*. D-Carbamylase activity was found in various bacteria belonging to the genera of *Agrobacterium*, *Pseudomonas*, *Comamonas*, and *Blastobacter*. The genes of these two enzymes were molecularly cloned and the transformant *E. coli* was able to be used as the practical enzyme source. To keep D-carbamoylase stable in the repeated use, a random mutation technique was applied to the *Agrobacterium* D-carbamoylase. Three heat stable mutant enzymes were obtained and these mutations were used as replacements for the amino acid residue at His 57, Pro203, and Val236. These mutations were combined with one molecule and the mutant enzyme, yielding triple mutation His57Tyr, Pro203Glu, and

Val236Ala with a 19 °C higher heat stability than the wild type enzyme [25]. *E. coli* cells containing this mutant enzyme were immobilized and used for practical industral production of D-HPG with the simultaneous use of immobilized D-hydantoinase on line. The immobilized D-carbamoylase reactor is able to be used for one year without any supply of new enzyme.

The enzymatic process of D-HPG production was started in 1980 in Singapore, and the immobilized D-carbamoylase reactor was introduced in 1995. The amount of production of D-HPG by this method is around 2000 tons/year [26].

10 Hydroxy-L-Proline

trans-4-Hydroxy-L-proline or *cis*-3-hydrosy-L-proline is produced from L-proline by the respective actions of L-proline 4-hydroxylase or 3-hydroxylase. The other substrate 2-oxoglutamate is supplied from glucose added to the reaction mixture:

$$\text{L-Pro} + \text{2-oxo-glutarate} + O_2 \xrightarrow{\text{4-hydroxylase}} \text{4-OH-Pro} + \text{succinate} + CO_2$$

$$\text{L-Pro} + \text{2-oxo-glutarate} + O_2 \xrightarrow{\text{3-hydroxylase}} \text{3-OH-Pro} + \text{succinate} + CO_2$$

trans-4-Hydroxy-L-proline is a component of animal tissue protein such as collagen and was extracted from collagen after hydrolysis with strong acid. The discovery of L-proline hydroxylases made the microbial production of hydroxyproline possible.

Ozaki et al. developed a specific hydroxyproline detection method with high performance liquid chromatography and screened microbial proline hydroxylase [27]. L-Proline 4-hydroxylase was found in some etamycin-producing actinomycetes belonging to the genera *Streptomyces*, *Dactylosporangium*, or *Amycolatopsis* [28]. L-Proline 3-hydroxylase was found in some telomycin-producing actinomycetes belonging to the genera *Streptomyces*, and in bacteria belonging to *Bacillus* [29].

The genes of these proline hydroxylase are molecularly cloned in *E. coli* cells and the cells overexpressing the enzyme were used as the enzyme source in the industrial process of L-hydroxyproline production. The genes obtained from actinomycetes proved somewhat difficult to be highly expressed in *E. coli* cells, and the genetic codens corresponded to the *N*-terminal of the enzyme protein were changed to match the codon usage in *E. coli.* Furthermore, the promoter of *trp* operon was introduced doubly at the promotor site of the gene in the plasmid to achieve the overexpression. These transformants expressed a 1400-times higher activity of 4-hydroxylase and a 1000-times higher activity of 3-hydroxylase in comparison with the original strain.

2-Oxoglutarate, one of the substrates of hydroxylation, is supplied from glucose in the reaction medium via the EMP pathway and TCA cycle in *E. coli* and the product succinate is recycled. The mutant strain of *E. coli* which lacks L-proline-degrading enzyme was obtained and used for the host cells of production of L-hydroxyproline.

Using *E. coli* cells in L-proline production as the host cells, the direct production of L-hydroxyproline from glucose became possible. In this case, the derepressed genes of L-proline biosynthetic pathway were introduced into *E. coli* cells together with the gene of L-proline hydroxylase [30].

The industrial production of *trans*-4-hydroxy-L-proline started in 1997. 4-Hydroxy-L-proline is useful as a chiral starting material in chemical synthesis and as a starting material for medicines, cosmetics, and food additives.

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Development of Biotechnology in India

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India has embarked upon a very ambitious program in biotechnology with a view to harnessing its available human and unlimited biodiversity resources. It has mainly been a government sponsored effort with very little private industry participation in investment. The Department of Biotechnology (DBT) established under the Ministry of Science and Technology in 1986 was the major instrument of action to bring together most talents, material resources, and budgetary provisions. It began sponsoring research in molecular biology, agricultural and medical sciences, plant and animal tissue culture, biofertilizers and biopesticides, environment, human genetics, microbial technology, and bioprocess engineering, etc. The establishment of a number of world class bioscience research institutes and provision of large research grants to some existing universities helped in developing specialized centres of biotechnology. Besides DBT, the Department of Science & Technology (DST), also under the Ministry of S&T, sponsors research at universities working in the basic areas of life sciences. Ministry of Education's most pioneering effort was instrumental in the creation of Biochemical Engineering Research Centre at IIT Delhi with substantial assistance from the Swiss Federal Institute of Technology, Zurich, Switzerland to make available state-of-the-art infrastructure for education, training, and research in biochemical engineering and biotechnology in 1974. This initiative catalysed biotechnology training and research at many institutions a few years later.

With a brief introduction, the major thrust areas of biotechnology development in India have been reviewed in this India Paper which include education and training, agricultural biotechnology, biofertilizers and biopesticides, tissue culture for tree and woody species, medicinal and aromatic plants, biodiversity conservation and environment, vaccine development, animal, aquaculture, seri and food biotechnology, microbial technology, industrial biotechnology, biochemical engineering and associated activities such as creation of biotechnology information system and national repositories. Current status of intellectual property rights has also been discussed. Contribution to the India's advances in biotechnology by the industry, excepting a limited few, has been far below expectations. The review concludes with some cautious notes.

Keywords. Biochemical engineering, Biotechnology education, Plant biotechnology, Animal biotechnology, Medical biotechnology, Food biotechnology, Environmental biotechnology, Industrial biotechnology

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List of Abbreviations

AIIMS	All India Institute of Medical Sciences
Bt	*Bacillus thuringiensis*
CBT	Centre for Biochemical Technology
CCMB	Centre for Cellular and Molecular Biology
CDFD	Centre for DNA Fingerprinting and Diagnostics
CDRI	Central Drug Research Institute
CFTRI	Central Food Technological Research Institute
CIMAP	Central Institute of Medicinal and Aromatic Plants
CMC	Christian Medical College
CPRI	Central Potato Research Institute
CSIR	Council of Scientific and Industrial Research
CSRTI	Central Sericultural Research and Training Institute
DAE	Department of Atomic Energy
DBT	Department of Biotechnology

DST	Department of Science & Technology
ELISA	Enzyme Linked Immunosorbent Assay
ETT	Embryo Transfer Technology
FSH	Follicle Stimulating Hormone
GOI	Government of India
GV	Granulosis Virus
HIV	Human Immunodeficiency Virus
IARI	Indian Agriculture Research Institute
IBR	Infectious Bovine Rhinotracheitis
ICAR	Indian Council of Agricultural Research
ICGEB	International Centre for Genetic Engineering & Biotechnology
ICMR	Indian Council of Medical Research
IFCPAR	Indo-French Centre for Promotion of Advanced Research
IHBT	Institute of Himalayan Bioresource Technology
IICB	Indian Institute of Chemical Biology
IISc	Indian Institute of Science
IIT	Indian Institute of Technology
IMTECH	Institute of Microbial Technology
ISBC	Indo-Swiss Collaboration in Biotechnology
IVRI	Indian Veterinary Research Institute
JNU	Jawaharlal Nehru University
MDR	Multi Drug Resistance
MKU	Madurai Kamraj University
MOU	Memorandum of Understanding
NARI	National AIDS Research Institute
NBRI	National Botanical Research Institute
NCCS	National Centre for Cell Science
NCL	National Chemical Laboratory
NDDB	National Dairy Development Board
NDRI	National Dairy Research Institute
NEERI	National Environmental Engineering Research Institute
NICD	National Institute of Communicable Diseases
NICED	National Institute of Cholera and Enteric Diseases
NII	National Institute of Immunology
NPV	Nuclear Polyhedrosis Virus
ORF	Original Replicating Factor
PGIMER	Post Graduate Institute of Medical Education and Research
RAPD	Random Amplified Polymorphic Deoxyribonucleic acid
RFLP	Restriction Fragment Length Polymorphism
RRL	Regional Research Laboratory
SDC	Swiss Agency for Development and Cooperation
SFIT	Swiss Federal Institute of Technology
TERI	Tata Energy Research Institute
TNAU	Tamil Nadu Agricultural University
UDCT	University Department of Chemical Technology
UDSC	University of Delhi, South Campus
UNDP	United Nations Development Programme

1 Introduction

Today India is in severe physical stress under a fast growing population, unmanaged decay of environment, rapid destruction of forest cover, inadequate health-care, malnutrition, poor health care facilities, damage of agricultural land, accumulating *xenobiotics* etc. It is ironic though that most of these maladies are amenable to remedies with selective application of available knowledge of biotechnology. India has generated a number of answers which are being implemented with joint efforts of appropriate Government agencies, scientists/technologists working at academic and research institutions and industry. During the pre-independent era (prior to 1947), the scientists and academics working in their respective fields were basically involved in a search for knowledge for self-satisfaction and earning their livelihoods with funds coming from the public exchequer. There was hardly any involvement of industry in these efforts; planning of need-based research in any sector for economic and social change was completely absent. Administration and bureaucracy were tuned primarily to keep law and order and the manpower needed to meet the administrative requirements were trained accordingly with minimum inputs of intelligent workforce. There were, however, extraordinary men teaching science at the Universities who rose to the pinnacle of success by their own intellectual strength in all fields of sciences like physics, chemistry, mathematics and astronomy despite many difficulties. Through the 75 years covering the fourth quarter of the nineteenth century till the middle of the present century, India produced many world class thinkers and persons of eminence in science and several of them became members of The Royal Society, London as elected Fellows in recognition of their original contributions. One outstanding example was the scientist J. C. Bose, a brilliant radio-physicist, who later changed over to study botany and in his discovery he quantified the plants' ability to respond to electrical signals and stimulated the perceived irrelevance of so-called differences between the living and the inanimate. Studies in biology, botany, zoology, and microbiology were generally confined to classical teaching of systematics.

This review covers, besides the infrastructure, centres of excellence and specialized facilities, sectors like education and training, environment, plant, animal, medical, food, and industrial biotechnology, as well as the country's efforts to promote links between industry and research institutions in biotechnology. The current status of India's pursuits in biotechnology or joint ventures with multinational cooperation with proven strength in biotechnology, with a few significant exceptions, is clearly far from narrowing the gap between the country's needs and the given opportunities.

Based on the available reports dealing with biotechnology research projects and creation of centres and facilities initiated after 1987 – 88, there appears an endless lists of projects funded by the Council of Scientific and Industrial Research (CSIR), Department of Science and Technology (DST) and, by far the largest, the Department of Biotechnology (DBT), Government of India. First, it is often difficult to distinguish between biology and biotechnology projects and second, project management set-up as not being structured, there is no way one can

comfortably determine the lines between the start and finish of the project and thus effective utilization of the results generated by the them. Quality research conducted in a number of world class centres is likely to make breakthroughs in the near future. These centres are in constant and active pursuit of excellence.

The review concludes with some comments.

2 Education, Training, and International Collaboration

While taking the first step towards formulating an appropriate national policy to build up biotechnology, the basic needs for adequate scientific manpower development were clearly recognized and funds for initiation of research were budgeted. Department of S&T under the central Ministry of Science and Technology constituted a National Biotechnology Board (NBTB) in 1982 at a time when the International Union of Pure and Applied Chemistry under ICSU accepted the decision of its constituent Commission on Fermentation to change the theme of its four yearly series of International Fermentation Symposium to International Biotechnology Symposium and to hold the 7th Symposium at New Delhi in 1984, for the first time in a developing country. Both IUPAC's decision and the Government of India's initiative augured well. In the same year, the 4th International Genetics Congress was also held at New Delhi. In consideration of hope and expectation that the developing countries might become significant shareholders of the profits of biotechnology R&D, UNIDO also took the initiative of establishing an International Centre for Genetic Engineering & Biotechnology (ICGEB) and one of its two components was established at New Delhi in 1986. Soon the NBTB was converted into a new Department of Biotechnology (DBT). These four significant events laid the foundation of the new biotechnology initiative in India.

On the education and training front, historically the B. Tech. program in Food Technology and Biochemical Engineering started in 1964 at Jadavpur University, Calcutta and at H. B. Technological Institute, Kanpur mainly to cater to the needs of the processed food industry. A program on Food and Fermentation Technology also began at the University Department of Chemical Technology, Mumbai at the same time. With substantial contents of fermentation and biochemical engineering, these centres began offering first degree programs in the discipline. The growth process of biotechnology through such programs was, however, found to be insufficient. Subsequently, an academic training and research program in biochemical engineering was initiated at IIT, Delhi in 1969. Since the Chemical Engineering Department, Jadavpur University had introduced an elective course in Biochemical Engineering in 1958 for the first time, a workshop celebrating twenty years of Biochemical Engineering Training and Research in India was jointly held at Jadavpur in 1978 [1]. The initial growth of biochemical engineering at IIT, Delhi was catalyzed by substantial scientific and technical support from the SFIT, Zurich which began in 1974 and was phased out in 1985. Both Prof. A. Fiechter (SFIT, Zurich) and Prof. T.K. Ghose (IIT, Delhi) had committed key role in this very first collaboration with SDC to seed an academic foundation of biotechnology in India. It gradually evolved into a world

class Centre of Biochemical Engineering Research (BERC) that finally led to the establishment of the first academic Department of Biochemical Engineering & Biotechnology in 1993 initiated six years ago. It stood up as a role model of Human Resource Development efforts in biotechnology. Substantial grant from UNDP, initially planned with UNESCO in December 1982 to augment the assistance from SDC was finally in place in early 1989. All these supports plus the grant and prompt clearance from the Ministry of Education and Culture of proposals of training of faculty staff at top universities around the world as well as rapid creation of modern infrastructure with UNDP support helped establish an excellent base for biochemical engineering training in India. In 1986, the Department of Non-Conventional Energy Sources, Ministry of S&T, approved nearly Rs. 16 million grant for BERC to establish a pilot plant facility for scale-up studies in the biochemical rendering of lignocellulosic residues to ethanol and coproducts based on data and results of doctoral and M.Tech. thesis work done at BERC between 1972 and 1986. This facility is currently used for large scale demonstration of bench scale data of some bioprocess systems. The 11 years of the pioneering Indo-Swiss cooperative program in India served not only as a role model of cooperation in S&T between two countries but it also helped many other institutes and universities to initiate similar programs at postgraduate levels.

The next phase of the ISCB began in 1988 and four new Indian scientific institutions were inducted into it. In 1995, a project review began and two more partners were integrated. The overall objective of the ISCB program set out now constituted enhancement of sustainable scientific and technological capabilities of the R & D institutions in the network for product development and technology transfer. More importance was given to a few criteria, applied to project selection, such as:

- Scientific quality, significance and feasibility
- Joint research between Indian and Swiss partner institute
- Feasibility of technology transfer and possibility of commercialization
- Legal and ethical aspects
- Compliance with the guidelines of the SDC and the DBT

The intensity of collaboration between Indian and Swiss partners differs from case to case. These are considered as Indian projects with largely Swiss support. Within this context, the broad area of biotechnological issues covered by the current ISCB becomes clearer. Projects not only pertain to the area of human health, animal husbandry, microbial processes, and products for agriculture, but also to the pharmaceutical industry.

While the program grew steadily in terms of objectives and financial volume, neither the legal framework nor its organizational set-up changed substantially. On the Swiss side, a full time management body consisting of one or two scientist(s) and one administrative staff unit were responsible for the implementation of the program and the management of SDC funds. An advisory committee supports the ISCB management in its activities. The Joint Project Committee (JPC) meets once every year to review the progress. Projects are funded by two different flows: on the Indian side, financial sanctions are

directly extended to each project by DBT while SDC resources are channeled through the ISCB management. Cost of the program are shared between SDC and DBT according to the bilateral agreement. The cumulative SDC contribution since 1988 has reached approximately 10 million Swiss Francs, out of which about 75% were project related. A major part of these project related funds (about 65%) was used for equipment, chemicals, and journals. At the end of the present phase, the cumulative Indian contribution to the individual projects amounted to less than 10% of the Swiss contribution (Fiechter, personal communication). Although Indo-Swiss collaboration in biotechnology has been very effective during the last 25 years, it is difficult to pin down its exclusiveness because the GOI's contribution in the creation of infrastructure and human resource development constituted a substantial part.

Following enactment of DBT, a number of universities and scientific institutions were given financial assistance to create essential facilities to conduct biotechnology training programs at several levels like M.Sc. (four semester), M.Tech. (three semester) and Ph.D. with studentship, and to provide academic training of faculty at many universities abroad as well as training of technicians in selected laboratories in the country. Today almost all universities, IITs, and the Indian Institute of Science, Bangalore offer excellent training in biotechnology. Most of the required financial supports come from DBT for biotechnology R & D and from DST for basic research in life sciences. Other agencies such as ICAR, ICMR, and CSIR have in-house manpower training programs in their respective disciplines. DBT has also created a few autonomous research institutes such as NII, New Delhi, NCCS, Pune, and CDFD, Hyderabad, and additionally developed infra-structural facilities at various institutes/ centres which provide inter alia training in specialized sectors of biotechnology.

Based on the total budget allocations mentioned in the DBT Annual Reports of the first year (1987–88), and the most recent one (1997–98), the Ministry of S&T's continued interest in the development of biotechnology in India can be assessed (Table 1) [2, 3].

Table 1. Major sectors of investment in biotechnology by DBT [2, 3]

Sector	Investment (Million Rupees)	
	1987–1988	1997–1998
Education and Training	54.0	62.5
Scientific Research	193.4	519.5
Creation of autonomous institutes, centres and investment in public sector undertaking in biotechnology.	82.0	336.7
Total	329.4	918.7

Education and training programs in various sectors of biotechnology currently in operation with DBT funding are:

- Two-year post-doctoral research programs at (a) IISc, Bangalore, (b) CCMB, Hyderabad, (c) Bose Institute, Calcutta, and (d) IARI, New Delhi; total intake 45
- Post-M.D./M.S. Certificate course (Medical Biotechnology) at AIIMS, New Delhi and PGIMER, Chandigarh; total intake 8
- Five-year Integrated M. Tech. in Biochemical Engineering and Biotechnology at IIT, Delhi (since 1989) ; intake 30
- Five-and-a half-year M.Tech. in Biotechnology at IIT, Kharagpur (since 1995), intake 10
- One-and-a-half-year M.Tech. (Biochem. Engineering) at Jadavpur University, Calcutta, intake 5
- One-and-a-half-year M.Tech. (Industrial Biotechnology) at Anna University, Chennai, intake 10
- Two-year M.Sc. (General) in Biotechnology at seventeen universities (including one at IIT, Bombay); total intake 214
- Two-year M.Sc. (Agricultural Biotechnology) at three universities; total intake 30.
- Two-year M.Sc. (Medical Biotechnology) at AIIMS, New Delhi; intake 10
- Two-year M. Vet. Sci. (Animal Biotechnology) at two universities; total intake 25
- Two-year M.Sc. (Marine Biotechnology) at Goa University, intake 10
- Diploma in Bioinformatics at MKU, Madurai, intake 10
- Technician Training program at MKU Madurai and Sri Venkateshwara College, New Delhi; total intake 10

Besides the aforesaid, almost all universities are offering courses in Life Sciences, Biochemistry, Biophysics, Molecular Biology, Genetics, Microbiology, Zoology, Botany, and Chemical Engineering, leading to degrees in respective disciplines.

According to a report on Planning Biotechnology Manpower in India [4], the majority of trained personnel are engaged in three principal areas: (a) R & D, (b) Production, and (c) Quality Control. The survey also indicates that in medical, agricultural, and allied establishments, the number of trained R&D scientists far exceeds production personnel, similar to what is seen generally in countries like USA, Europe, and Japan. However, given this position it may be mentioned that in any of these sectors, contributions from the trained personnel to industrial biotechnology appear incompatible. The reasons include (a) migration to USA and Europe of approximately 50% of highly qualified persons after having acquired world class training in India [5], (b) industry's hesitation to develop or absorb indigenously produced know-how, (c) reluctance of blue chip multinational biotechnology corporations getting their feet firmly fixed in India, and (d) the prevailing confusion of how to handle the Intellectual Property Rights of biotech products.

Projected manpower need (sector wise) in the year 2000 has been estimated as follows [4]:

- Medical and health care 1010 – 1090
- Agriculture and allied field 1230 – 1450
- Chemical sector (commodity and high value) 440 – 473
- Bioinstrumentation, process hardware and engineering 400 – 540

Additionally, a national network of biotechnology information exchange and retrieval covering ten Distributed Information Centres and twenty three Distributed Information Sub-centres has also been initiated by DBT in 1989 and subsequently augmented. The Apex centre located in the premises of DBT, New Delhi coordinates the global network activities. It provides bioinformatics and biocomputing services to the researchers engaged in biology and biotechnology R & D and manufacturing activities all over the country. The services include analysis of biological data, bibliographic information on published literature, software development for computationally intensive problems in biology such as molecular modeling and simulation, genome mapping, structure – function determination, structure based drug design, structure alignment and comparison, structure prediction, molecular evolution, gene identification, etc.

DBT has also been supporting a number of repositories for conservation of living organisms for various sectors of biotechnology such as agriculture, health-care, animal husbandry and industry. These are:

- Microbial Type Culture Collection at IMTECH, Chandigarh
- National Facility on Blue Green Algae Collection at IARI, New Delhi
- National Facility for Marine Cyanobacterial Germ Plasma Collection at Bharathidasan University, Trichy
- National Bureau of Plant Genetic Resources at IARI, New Delhi
- Repository on Filarial Parasites and Reagents at Mahatma Gandhi Institute of Medical Sciences, Wardha
- Repository on Medicinal and Aromatic Plant Materials, at CIMAP, Lucknow
- Repository on Cryopreservation of Blood Cells at Indian Institute of Haematology, Mumbai

Consolidation of these facilities throughout the country continues to be DBT's high priority efforts.

DBT has also established international collaboration with several countries in areas other than education and training. During the period 1987 – 1998, more than 20 agreements in biotechnology between India and other countries were signed. Notable amongst them are Switzerland (with Anna University, Chennai, NEERI, Nagpur; M.S. University, Baroda and Indian Veterinary Research Institute, Izatnagar & Bangalore), USA, China, France, Germany, UK, Sweden, Israel, G-15 countries, Russia and a few others. Most benefits of these international efforts were, however, confined to a few Indian Universities and national laboratories where infrastructural facilities and financial assistance were provided by the DBT and other international S & T agencies including UN bodies.

IFCPAR is an instrument of scientific collaboration in almost all fields of basic sciences and in a few engineering sciences which was jointly instituted by the Governments of India and France in 1987. The centre is an autonomous body under the joint control of DST and the French Ministry of Foreign Affairs. Its budget is shared equally by the two governments and all decisions are taken together. Joint seminars, workshops, and symposia on topics of current interest are organized under the advice of the centre's Scientific Council, having eminent members drawn from both India and France. The centre is managed by two co-chairpersons, one from each country. Review of progress of projects and close

interaction between scientists of both the countries are a regular feature of the centre's activities.

Thrust areas of research in life sciences and biotechnology include molecular and cell biology, genetics, and genetic engineering, ecology and separation sciences. During 1997 – 1998 twelve projects in these areas were supported, out of which five were completed and seven were in progress. Some of the project areas and collaborating partners in India and France are briefly cited below:

1. Prof. Kiran Kuduria, AIIMS, New Delhi India and Prof. Mare Fillous, Institut Pasteur, Paris, on Molecular Studies of Sex Determination (on going)
2. Dr. Vatsala M. Doctor,Breach Candy Medical Research Centre, Mumbai, and Prof. Amu Therwath, Université Paris VII France on Breast Cancer in High Risk Ethnic groups (completed)
3. Prof. G.Metha, University of Hyderabad, Hyderabad and Prof. H Chanon, Universite d' Aix-Marseille III, Marseille, France on Design, Mechanistic Studies and Biological Activities for Photodynamic Therapy of Tumors, Cells and Leukemias (completed)
4. Prof. Ravi Parkash, Maharishi Dyanand University, Rohtak, India and Dr. Jean R. David, Laboratorie de Populations, Genetique et Evolution, Gif-sur-Yvette, France on Ecological and Evolutionary Genetics (completed)
5. Dr. Malathi Lakshmikumaran, TERI, New Delhi, and Prof. Michael Delserry, Laboratorie de Physiologie et Biologie Moléculaire Vegetables, Université de Perpignan, Perpignan, France on Mapping of *Brassica* genomes (completed)
6. Dr. R. Tewari, NCL, Pune, and Prof. Henri Grosjean, Laboratorie d'Enzymologie et de Biochemie Structurales, Gif-sur-Yvette, France on Post-transcriptional Modifications of Biological Functions of *E. coli* (completed)
7. Dr. J. Gowrishankar, CCMB, Hyderabad, and Prof. Henri Bue, Institut Pasteur, Paris on In-Vitro Studies on Osomotic Regulation of proU Transcription (ongoing)
8. Dr. Ranju Ralhan, AIIMS, New Delhi and Dr. Bohdan Wasylyk, Universite Louis Pasteur on Genetic Alterations in Pre-cancerous and Cancerous Oral Lesions (ongoing)
9. Dr. D. P. Kasbekar, CCMB, Hyderabad and Dr. Godeleine Faugeron, Institute Jacques Monod, Universite Paris VII, Paris on Isolation of Genes Encoding Sterol Biosynthetic Enzymes from *Ascobolus immersus* (ongoing)
10. Dr. Pradip Sinha, Devi Ahilya University, Indore, and Dr. Jean Maurice Dura, Université Paris XI, Orsay, France – On Transregulation of Homeotic Genes in *Drosophila* (ongoing)
11. Prof. G. P. Agarwal, IIT-Delhi, New Delhi and Dr. Pierre Aimar, Université Paul Sabatier, Toulouse, France on Transmission of Proteins through Porous Membranes (ongoing)
12. Sanjay N. Nene, NCL Pune and Prof. Bharat Bhusan Gupta, Université de Franche Copte' Belfort, France on Fouling of Membranes in the Clarification of Sugar Cane Juice (ongoing)
13. Prof. Raghavendra Gadagkar, IISc, Bangalore and Dr. Christian Pecters, UPMC, Paris on Behavioral Ecology of some Indian Ants (ongoing)

Funds provided to the five completed projects to 1998 amounted to Rs. 9.2 million and FF 2.9 million. The eight ongoing projects were allotted Rs. 16.4 million and FF 3.7 million. The authors of the above-mentioned projects have made several good publications cited in [6–17]. These reveal results of studies on molecular cloning and characterization of extracellular sucrase genes of *Zymomonas mobilis*, SACB, and SACC genes encoding levansucrase and sucrase from a gene cluster in *Zymomonas mobilis*, remarkable variety of plant RNA virus genomes, monoclonal antibodies in the study of architecture of plant viruses and bacterial transformation using microwave radiation [9–13].

3 Plant Biotechnology

Agriculture is the most important sector of the Indian economy contributing approximately 40% to national income. Through induction of advanced crop production technologies relating to high yielding cultivars, increased use of fertilizers and pesticides, and expansion in irrigation facilities, it has been possible to achieve a target of approx. 200 million tons per annum of food grain production. In order to meet the demands of continuously increasing population, biotechnological inputs are being made to claim all round sustained improvements in the agriculture sector for food security. As an apex organization, the ICAR provides support for overall agricultural development through its 45 central research institutes, 30 national research centres, and other services. The most laudable achievement during the course of the last three decades included attaining the second largest production of wheat and rice in the world, the largest production of fruits, doubling of oilseeds production in the last ten years and development of hybrids of a few major crops for increased productivity. The Council has given appropriate emphasis on environmentally sustainable agriculture through accelerated efforts on R & D. In the area of cereal production three quarters of the total cropped area for cereals in India is under high yielding varieties. Of the total cropped area in 1995–1996, high-yielding wheat and rice covered 92.4% and 77.3% respectively. Rice production would promptly double if yields were on a par with several Asian rice growing countries (India 28.8, Vietnam 36.4, Japan 60.1, China 60.2, and USA 62.7 hundred kg ha^{-1} and this would bring India very near to Japanese and Chinese yields, the two highest in Asia. Specifically, in rice output India ranks 2nd in the world but yield-wise only 54th. Massive efforts in biotechnology such as use of biopesticides, biofertilizer, improved seeds, and exposure of farmers to the elements of biotechnology backed by non-partisan political decisions may enable India to do much better than her current performance. However, resistance against the use of genetically modified seeds in Indian agriculture, already visible, may intensify by the environmental activists fearing widespread damage to the country's biodiversity already under stress.

Besides covering the important biotechnological inputs made in agriculture, this section also provides a brief account of advances being made in other areas of plant biotechnology, namely, conservation of germ plasm, micropropagation of tree and woody species for forest conservation, medicinal and aromatic plants etc.

3.1 Crops

Priority crops include rice, wheat, rapeseed, mustard, chickpea, mungbean, sorghum, peas, and cotton. Different aspects of biotechnology methods concerning these crops are being studies at NCL, Pune; M.S. University, Baroda; JNU, New Delhi; IARI, New Delhi; Bose Institute, Calcutta; TERI, New Delhi; Delhi University and ICGEB, New Delhi amongst others. Six centres have been specifically identified and supported to work on molecular biology aspects of plant crops, namely, JNU, New Delhi; TNAU, Coimbatore; MKU, Madurai; Osmania University, Hyderabad; Bose Institute, Calcutta and NBRI, Lucknow. At these places research is being carried out on several crops on transformation, plant vector development, molecular aspects of *cis* and *trans* elements or factors, storage proteins, control mechanisms at gene level upstream regulatory elements, molecular biology of chloroplast and mitochondria, characterization of tissue-specific promoters/genes in relation to male sterility, and exploitation of heterosis [18].

The achievements over the years and the current research activities on the food crops at a few selected R & D institutes are briefly described below.

In plant tissue culture, India has always been at the forefront. A novel technique of test tube fertilization was developed at Delhi University to overcome incompatibility in plants exhibited in wild crossing. This technique developed in the 1960s is being employed in many laboratories all over the world. Another landmark achievement in plant breeding and genetics related to production of haploids through another culture of Datura for the first time to improve crop plants; this and development of triploid plants through endosperm culture were also first created at Delhi University in the 1970s. Triploid plants produce seedless, juicy fruit, an example being triploid watermelon. Protocols have been developed for clonal multiplication of hundreds of plant species which include trees, medicinal and aromatic plants, and endangered species from several laboratories across the country. Flowering of bamboo, which is a rare phenomenon, was demonstrated by NCL, Pune scientists in the 1980s. (Guha Mukherjee, personal communication).

In wheat, the signal transduction pathway leading to somatic embryogenesis following auxin applications has been worked out at UDSC, New Delhi. Further characterization of various aspects of somatic embryogenesis is currently underway. These systems are also being utilized for *Agrobacterium*-mediated transformation. In another project on genetic engineering of plants tolerant to abiotic stresses, nearly 100 proteins up/down regulated in rice seedlings in response to salinity, desiccation and low and high temperature have been characterized. A 104-kDa protein has been characterized by amino acid sequence analysis of three different tryptic peptides. Interestingly, most of the protein alternatives were found to be similar in flooding situations, sensitive and tolerant types indicting that flooding may not involve a very large number of genes [2].

Transgenic plants are those plants in which functional genes have been inserted in their genomes. With advances in recombinant DNA methods and transformation procedures, it is possible to transfer genes into crop plants from

unrelated plants, microbes, and animals. Availability of efficient transformation systems for crop species is of immense interest in biotechnology. However, the application of this technology to rational plant-improvement is currently limited by a shortage of cloned genes for important traits. Taking note of this, Prof. Asis Datta's group at JNU, New Delhi, a pioneering centre for biological research, reported two novel genes having direct bearing on nutritional status of crop and in turn human health, namely Amaranth seed protein, Am A1, and oxalate decarboxylase, OXDC. In an attempt to improve the nutritional quality, the coding sequence of amaranthus seed albumin (AmA1) was stably introduced into potato plant. The AmA1 protein is rich in all essential amino acids, including lysine, tryptophan, and also sulfur-containing amino acids, particularly, methionine. Its amino acid composition favorably corresponds to that of the World Health Organization's recommended protein standard for optimum human nutrition. The protein expressed was found to be stably accumulated in transgenic tubers. A significant increase in most essential amino acids was observed on amino acid analysis. Almost all of the essential amino acids increased by 3- to 20-fold. There was, however, no reduction or significant change in any of the major tuber proteins. Unlike most storage proteins, AmA1 protein proved to be a non-allergen. These findings suggest that the AmA1 protein is a potent candidate for improvement of nutritional quality of other important crop plants which are otherwise deficient in one or other essential amino acids. The gene OXDC is responsible for the degradation of oxalic acid, which is harmful in many cases. Much of the oxalate from animals including humans originates from the oxalate ingested with plant material. Some green leafy vegetables (e.g., amaranthus, spinach, rhubarb) are rich sources of vitamins and minerals but they contain oxalic acid as a nutritional stress factor. Besides, at least two other instances can be cited where oxalic acid is involved in an indirect manner. In one case, the production of oxalic acid is an important attacking mechanism utilized by *Whetzelinia sclerotiorum*, a fungus that causes serious damage to crops like sunflowers. Oxalic acid accumulates in the infected tissue early in pathogenesis, and its concentration increases during the time the pathogen is colonizing the host tissues. The accumulation of oxalic acid in leaves causes symptoms of wilting and eventually leaf death. Thus, oxalic acid functions as a mobile toxin that moves from the base of stems to xylem sap and leaves. In another case, consumption of *Lathyrus sativus* causes neurolathyrism, which is characterized by spasticity of leg muscles, lower limb paralysis, convulsions and death. *L. sativus* is a protein-rich hardy legume that grows under extreme conditions such as draught and water-logging and does not require complex management practices. The neurotoxin ODAP is present in various parts of the plant. ODAP synthesis is a two-step reaction in which oxalic acid is an essential starting substrate. It acts as a metabolic antagonist of glutamic acid, which is involved in transmission of nerve impulses in the brain. Hence, despite its rich protein content, the legume cannot be used as a food source. As part of a long-term program to develop transgenic plants with low oxalic acid content, the coding region of OXDC gene was stably introduced in *Nicotiana*. The transgenic lines showed high-level expression of this protein. Both transgenic *Nicotiana* and tomato plants also exhibit significant resistance to fungal infection by *Sclerotinia sclerotiorum* in

vivo. The next step is to develop transgenic *Lathyrus* with very low levels of the above-mentioned neurotoxins [19–23].

Among several factors which affect yeast to mycelial transition in *Candida albicans*, various nutrients, namely sugars, amino acids, and other nitrogen sources etc., play an important role. Prof. Rajendra Prasad's group, also in JNU, New Delhi is ascertaining the molecular mechanisms of transport of nutrients (particularly the amino acids) and xenobiotics (drugs) in yeast. The group has purified and functionally reconstituted proline and arginine permeases into liposomes and demonstrated that these permeases, upon reconstitution, can mimic transport features of intact cells. Two ORFs of *C. albicans* have been identified and sequenced which upon expression complement *put*4 mutation of *S. cerevisiae*. The multidrug transporters. which are of two types, namely (a) ATP Binding Cassette (ABC) and (b) the Major Facilitator Superfamily (MFS), contribute to an increased efflux of cytotoxic compounds. In this regard, the characterization of multidrug resistance genes, *CDR1* (an ABC type of *Candida* drug resistance gene), was an important step towards the development of effective chemotherapy and improved drug designing. *CDR1*, a homologue of human *MDR1*, is a 169.9 kDa transporter consisting of two homologous halves each comprising one hydrophobic region consisting of six transmembrane helices preceded by one nucleotide binding fold. *CDR1* confers resistance of a broad spectrum of drugs and the expression of *CDR1* is enhanced in fluconazole resistant clinical isolates of *C. albicans*. Apart from effluxing drugs, which is driven by ATP hydrolysis, it can efflux human hormones like β-estradiol which could be one of the physiological substrates. The over-expression of *CDR1* in presence of steroids like progesterone and β-estradiol supports the above observation. Recently, it has also been shown that *CDR1* is a general phospholipid translocator which could flop phospholipids from cytoplasmic monolayer to exterior monolayer. This function could be the normal physiological function of *CDR1*. These functions of *CDR1* are affected by fluidity status of the plasma membrane. *CaMDR1* (Benomyl resistance gene *Ben*^{r}) and its mutant alleles have recently been identified. *CaMDR*, a MFS, differs from *CDR1* in that the drug efflux is driven by a proton gradient and not by ATP hydrolysis. Over-expression of *CaMDR1* in some of the fluconazole resistant clinical isolates suggests its involvement and points to multiple mechanism of drug resistance in this pathogenic yeast [24–26].

Studies at the Bose Institute, Calcutta on inositol metabolism in relation to salinity tolerance in rice indicated that activity of cytosolic and chloroplast 1,6-bisphosphatase declines in the sensitive varieties. It was also confirmed that activity of purified enzyme remained unaltered in vitro in wild rice *P. coartata*. For cloning of molecular markers involved in salt tolerance and their over-expression to enhance salt tolerance, PCR amplification of cDNAs is in progress. Studies on improvement of aromatic rice, development of mapping population through double haploids, are also underway for aroma genes. Efforts are also underway for tagging three quality traits (protein content, preharvesting sprouting tolerance, and seed size) in hexaploid wheat. The parental analysis has been initiated using three different approaches, namely RFLP, microsatellite, and RAPD to detect the number of polymorphic enzyme producing combinations.

Work on DNA fingerprinting and genetic diversity analysis of tetraploid wheat in relation to evaluation of glutenin and gliadin polymorphism in durum, evaluation of β-carotene, and development of mapping populations is also underway [2, 27].

Naturally occurring isolates of *Bacillus thuringiensis* are known to produce crystalline inclusions during sporulation. These inclusions consist of insecticidal polypeptides active against specific insects. Genes coding for these polypeptides have been expressed in plants. It has been observed that those genes are expressed poorly because of the presence of destabilizing signals in toxin coding genes. Elimination of such sequences enhanced the level of expression of toxin polypeptides. A toxin coding gene (*cry* 1Ia5) devoid of such destabilizing signals has been identified and characterized at ICGEB, New Delhi, thereby allowing its adequate expression in transgenic plants [28]. The transgenic tobacco plants expressing native gene were completely protected against predation by *Heliothis armigera*. The results also demonstrate that novel insecticidal toxin coding genes already exist in nature which do not require extensive modifications for efficient expression in plants. Cry 1Ia5 toxin is also active against agronomically important pests, like *Plutella xylostella* (Diamond-back moth), *Leucenoides orbanalis* (Eggplant borer), and *Chilo partellus* (Spotted-stalk borer). In addition, scientists at ICGEB have cloned, sequenced, and expressed vegetative insecticidal protein (VIP) from an isolate of *B. thuringiensis*. Activity spectrum of VIP and cry 1Ia5 overlap in effectiveness against *C. partellus*. These two toxins are structurally unrelated and hence are likely to interact with different receptors on the mid-gut of susceptible insects. The combination of these toxins will be very beneficial in the pest management programs. The prospects of commercialization of these toxin-bearing constructs for making transgenic crop plants are being explored in collaboration with plant breeding companies [Chatterjee, personal communication]. Research efforts on development of disease-resistant crops are also underway at several institutes including IARI, New Delhi; Bose Institute, Calcutta; and MKU, Madurai.

High level expression of foreign genes has long been recognized for the conversion of plant cells into bioreactors to produce important agricultural, industrial, and pharmaceutical compounds. The spread of transgenes into wild relatives and other crops through cross pollination is also an important issue related to the environmental risks of genetically modified organisms. In this context, the ability to transform plastids, given the existence of multiple copies of chloroplast DNA in each plastid and the maternal inheritance of plastid genes, attracted the attention of geneticists to express foreign genes in the chloroplasts of higher plants.

ICGEB, New Delhi has been working on the expression of Hepatitis B surface antigen (HbsAg) in plants with the hope of claiming a vaccine in edible form. Also, the problems that are associated with the traditional vaccines such as storage, transportation, and administration may be overcome. The centre has developed transgenic tobacco plants expressing HbsAg in the chloroplasts. The expression levels are several hundred-fold higher than those previously reported using nuclear transformation. An immunomodulator displaying antiviral and antiproliferative properties (human gamma interferon, γ-IFN) suggest its

possible therapeutic use in the treatment for rheumatoid arthritis, atopic dermatitis, venereal warts etc. γ-IFN is known to express itself poorly when introduced into the nucleus of the tobacco plant. Introduction of the same gene into chloroplast has achieved increased expression by 50–60-fold as demonstrated by ICGEB, New Delhi. The results indicate the feasibility of high level production and purification of foreign proteins from plants [Chatterjee, personal communication].

Other important projects executed in several centres on edible vaccines include (a) expression of antigenic determinants of *Vibrio cholerae* in tomato or tobacco at UDSC, New Delhi; CBT, New Delhi; NII, New Delhi; and IMTECH, Chandigarh and (b) development of transgenic cabbage as edible expressing glycoprotein G of rabies virus at CIMAP, Lucknow; NDRI, Lucknow; and IVRI, Izatnagar [2].

In order to reduce dependence on chemical fertilizers, a number of organizations are promoting the application of vermiculture technology to restore and maintain long-term productivity of soil for increased crop production.

3.2
Biocontrol of Plant Pests

ICAR and several state agricultural universities have been conducting programs on control of major pests of crops. DBT considers the biocontrol network program as an important area; it provided nearly Rs 160 million to more than 60 R & D projects. The program aims at development of cost-effective, commercially viable technologies for the biocontrol agents like baculoviruses, parasitoids, predators, antagonistic fungi, and bacteria for use under integrated management of major pests and diseases of economically important crops. These efforts have promoted mass production techniques for several biopesticides, such as NPV of *Heliothis armigera*, NPV of *Spodoptera litura*, GV of *Chilo infuscatellus*, *Trichogramma*, (developed by IARI, New Delhi), and *Chrysopa*, *Trichoderma viride* (developed by RRL, Jammu). About 11,700 hectares area are now used for experimental farming of cotton, chickpea, tobacco, sugarcane, groundnut, sunflower, black gram, green gram, pigeon pea, and other pulses to establish the effectiveness of these biosubstances [Paroda, personal communication].

Progress has also been visible in the areas of microbial and other pesticides including transgenic Bt, insect cell lines, pheromones, and botanical pesticides. IARI has developed transformants using Bt genes in brinjal, tomato, cauliflower, and cabbage. Genes have also been mobilized in *Indica* rice – once thought to be a formidable task. CPRI, Shimla has developed transgenic potato which are under evaluation. The Indian private sector has developed transgenics in cotton (Mahyco-Monsanto), brassica, tomato, and brinjal (Pro-Agro-PGS Belgium) [Paroda, personal communication].

Five selected pheromones have been synthesized by simple processes for monitoring and control of insect pests of cotton, red gram, Bengal gram, maize, vegetables, potato, cabbage, sorghum, etc. Under the botanicals, the nematicidal efficacy of various neem products against the major groups of phytonematodes

has been established under field conditions. Extracts of leaves of *Melia azardirach* are more than 80% larvicidal against *H. armigera* and aphids. An effective method for the management of *Parthenium* in non-cultivated areas has been developed. A bioformulation of an antagonistic yeast (*Debaryomyces hansenii*) has been found useful for control of post harvest diseases of citrus and mango fruits [2].

3.3 Tree and Woody Species Tissue Culture

Tissue culture techniques applied to various tree and woody species continue to be developed at various institutions. The Neem tree, having high contents of azadirachtin and oil, has long been domesticated as an important part of the Indian ecosystem. Currently these are being mass produced at Dalmia Centre, Coimbatore. Micropropagation protocol for 16 mangrove species is claimed to have been perfected by M.S. Swaminathan Research Foundation, Chennai. Nearly 60,000 plants have been produced and field transferred. At Kerala Forest Research Institute, Trissur, an ELISA technique for detecting the presence of phytoplasma has been reported for identification of spike resistant sandalwood plants. The technique for mass production of disease-resistant sandal trees through somatic embryogenesis has also been standardized. Banaras Hindu University, Varanasi, NDRI, Lucknow, and NCL, Pune are collaborating to develop protocols for plantlet regeneration of a number of mango varieties through somatic embryogenesis. The process of synchronized maturation of embryos and hardening of plantlets in soil is under investigation. Similar work is in progress at other institutes for micropropagation of chrysanthemums, roses, orchids, pepper, spices (clove, nutmeg, cinnamon, etc.) tea, coffee, saffron, *Populus*, and bamboo. A UNDP assisted project on jute under the Ministry of Textiles to investigate characterization of jute germplasm and wide hybridization breeding, development of an enzymatic retting protocol, and development of transgenic jute resistant to diseases and insects is under way. The program is being coordinated by DBT at five institutions [2].

3.4 Medicinal and Aromatic Plants

A number of medicinal and aromatic plants are being micropropagated to conserve their germplasm and to harness their economic potential; one of these, *Taxus* sp., a source of anti-cancer drug taxol, is being simultaneously studied at NCL, Pune; RRL, Jammu; RRL, Jorhat; CIMAP, Lucknow; and IHBT, Palampur. The objectives include germ plasm conservation, mapping, and distribution of the plant at various locations, standardization of techniques for micropropagation, as well as establishing density plantations of *Taxcus baccata*, and biotransformation of taxane derivatives, namely 10-deacetyl baccatin, for production of taxol [29, 30]. CIMAP, Lucknow has developed high yielding varieties of *Catharanthus roseus, Commiphora wightii, Duboisia myoporoides and Glycorrhiza glabra* by genetic improvement. CFTRI, Mysore, and IIT, Delhi are actively

developing process details for production of secondary metabolites (conessine, podophyllotoxin, capsaicin, vanillin, etc.) by employing shear-sensitive plant cells in bioreactors in suspension and in immobilized form [29, 30].

3.5
Bioprospecting

India is a land of enormously rich biodiversity with two hot spots in the North-East Himalayas and South Western Ghats. In view of the biological wealth of these areas, a major initiative involving 13 collaborating institutions was launched on bioprospecting in 1997. Characterizing, inventorying, conserving biodiversity, and prospecting of different ecogeographical regions constitute its objectives. Department of Space is associated with this effort for remote sensing and satellite imaging of the identified areas for preparing biome maps. Institutes associated with the project are IISc, Bangalore; NCL, Pune; Botanical Survey of India, Delhi University; and IHBT, Palampur. Three National Gene Banks have also been set up for conservation of plant germplasm at following institutes: (a) National Bureau of Plant Genetic Resources, New Delhi, (b) CIMAP, Lucknow, and (c) Tropical Botanical Garden & Research Institute, Thiruvananthapuram [2].

CCMB, Hyderabad, was set up by CSIR in 1977 for research in basic studies in the frontier areas of modern biology and for exploring potential biotechnological applications. The centre also conducts investigations on osmoregulation in *E.coli,* prokaryotic transcription, peptide-membrane interactions, signal transduction, intracellular protein transport, eukaryotic gene regulation, tumor biology, biochemistry of cataract, mechanism of sex determination, mammalian reproduction, action of steroid hormones, host-pathogen interactions, and mathematical modeling of population dynamics etc. The centre has also been identified for research in several aspects of cellular control processes and molecular genetics of biotic and abiotic stress of plants dealing with aspects like: (a) intracellular sorting of proteins, (b) mechanism of nuclear transport of proteins, nuclear assembly, role of a novel class of nuclear phosphoproteins, (c) regulation of cell division cycle, role of an unusual protein tyrosine phosphatase which binds to DNA, (d) role of cell adhesion in modulation of cell cycle, (e) genetic analysis of spontaneous loss of virulence in *Xanthomonas oryzae*, a causal agent of bacterial leaf blight in rice, (f) genetic and molecular analysis of phosphate uptake and phosphate toxicity in *Arabidopsis thaliana*, and (g) elucidation of the mechanism of antibiotic resistance in cellular slime molds [2, 31].

4
Medical Biotechnology

One of the internationally acclaimed Divisions of Biological Sciences of the Indian Institute of Science, Bangalore consists of the Departments of Biochemistry, Microbiology & Cell Biology, Molecular Reproduction, Development and Genetics, Molecular Biophysics and Ecological Sciences. The Division has a good small animal facility, a Primate Research Laboratory and a Bioinformatics

Centre. It receives grants from almost all important S&T agencies in India. Around 50 faculty and 250 researchers (Ph.D. students and post doctoral students) are engaged in a wide range of basic biology and biotechnology [Vijayan and Padmanabhan, personal communication].

The major areas of research include DNA transactions, protein structure and function, receptors and signal transduction, protein engineering, reproductive endocrinology, developmental biology, and ecology. A wide variety of animal, plant, and microbial systems have been used in these studies. Major contributions in basic research include clarity in the understanding of the processes of meiotic DNA recombination, DNA recombination in λ phage and yeast, transcription studies using cytochrome P-450 in rat liver, silkworm tRNA, mom C virus genes, and cryptic genes in *E. coli*. The structure and interactions of lectins from different plant sources have been investigated. Important contributions have also been made on the structure of sesbania mosaic and related viruses and in areas of lectins. Polypeptide conformations in relation to transport across channels and membrane architecture, activities pertaining to DNA conformation, molecular modeling, protein design, and structural data analysis are all in progress. Reproductive biology forms an important component of research at IISc, Bangalore. Investigations on the genetic basis of development of plant and fungal systems, structure-function of many enzymes such as folate pathway enzymes, serine hydroxymethyl transferase, Type II restriction enzymes, glucoamylases, topoisomerases, uracil NA glycosylase, triosephosphate isomerase etc., molecular virology of rotavirus, rinderprest virus, and TMV are also in progress. Work on food and environmental allergens and immunological studies on a variety of infectious diseases and DNA vaccines have also been in progress. Information retrieval on biological diversity, social behavior, human ecology, ecodevelopment, climate change, and tropical forests have been undertaken. A major part of biomedical research activities in the Division falls under: (a) Infectious Diseases, (b) Drug and Molecular Design, (c) Genetic Disorders, (d) Gene Targeting, and (e) Genetic Diversity.

Examples of application oriented projects which got under way include: male fertility regulation; anti-tubercular drug screening using cloned gyrase from *M. tuberculosis*; heme biosynthesis in malarial parasite as a new drug target; pathogenesis of *M. tuberculosis* and Japanese encephalitis virus; diagnostics and vaccine candidate from rinderpest virus; inhibitor design; lectins in diagnostics; tissue culture of useful plants; transgenic plants with useful traits etc. [32–40].

Several of the studies mentioned have led to a significant level of interaction with industry. Nearly two dozen industries are involved in as many projects. While the studies are at various stages of development, specific achievements so far include: a peptide diagnostic kit for HIV (in the market); a pyrogen testing kit; DNA based test procedures for genetic disorders; a candidate rotavirus vaccine (clinical trial stage); a candidate DNA vaccine for rabies (clinical trial to start); recombinant hepatitis B vaccine (transferred to company and production to start in a few months); a diagnostic kit to identify snake bites (transferred); a functional allergy clinic; tissue culture protocol for sandal and forest trees; overexpression of proteins such as human growth hormone, FSH, and topoisomerases. The Division has close to 100 sponsored projects (supported by

various Government agencies) and around 30 projects involving support from industry [Vijayan and Padmanabhan, personal communication].

The National Institute of Immunology (NII), New Delhi created in 1986 is extensively engaged in studies to understand mechanisms of several immune systems in order to pursue creative solutions to a broad range of health problems of special importance to India. The institute pursues research in four major areas: (i) immunity and infection, (ii) gene regulation, (iii) molecular recognition, and (iv) reproduction and development. Serious global competence is being sought in each of these areas as measured by international peer reviewed publications. Notable contributions from the institute in the last five year (1993 – 98) dealt with programs on: (a) the elucidation of control mechanisms governing immune responses, (b) regulation of gene expression in a major industrial recombinant expression system, the baculoviruses, (c) structural analysis of molecular recognition and protein synthesis, (d) molecular design and physiological significance of various enzymatic systems, and (e) development of novel rationales for targeting therapeutics as well as immunomodulators [41 – 48]. The institute has so far been granted 20 foreign patents. Additionally, 16 Indian and 7 foreign applications are pending as of September 1998 [Basu, personal communication].

The institute also has a long-term strategic plan emphasizing excellence in rigorous fundamental research of high scientific merit, and pursuit of emerging leads for tangible public utility with useful entrepreneurial partnerships. NII has also been able to score a number of practical achievements, for example discovery of an immunotherapeutic adjunct that reduces the period of multidrug treatment of lepromatous leprosy down by a half or more. This know-how has been transferred to a major pharmaceutical house which is planning to produce and market the product under the trade name "Leprovac" [Basu, personal communication]. The institute claims to have validated the principle of contraceptive auto-vaccination in clinical trials for the first time in the world, thereby establishing a new path for the development of immuno-contraceptives. This vaccine is internationally patent-protected. Logistical concerns and variable responses are issues being addressed by ongoing research in this area before clinical use of the vaccines can be considered feasible. A new anti-cancer peptide product, MuJ-7, has been adopted as an R & D focus by a major Indian industry with substantial investment. NII has, nonetheless, developed and transferred to industries diagnostic test kit prototypes for detection of pregnancy, hepatitis B, typhoid, amoebic liver abscess, intestinal amoebiasis, *Streptococcus* A, HIV, and cattle embryo sexing, with limited commercial success so far.

The School of Life Sciences, JNU, New Delhi is another pioneering centre for biochemical research. One of the important projects being carried out relates to the study of molecular biology of the dimorphic pathogenic yeast *Candida albicans* with the aim of understanding the nature of virulent factors associated with the pathogenicity of this organism. With the discovery of AIDS, this commensal organism has gained immense importance with its ability to become systemic and invade a variety of tissues and organs in the human body. Prof. Asis Datta's group demonstrated the role of calcium, calmodulin, pH, and protein phosphorylation in morphogenesis of *C. albicans*, dimorphism being considered important for the virulence of this organism. This pathogenic yeast

colonizes on the mucous membrane rich in amino sugars and it has been observed that all pathogenic species of *Candida* can utilize *N*-acetylglucosamine as a sole carbon source thereby making this *N*-acetylglucosamine catabolic pathway important for pathogenicity. To study this pathway, the key enzyme *N*-acetylglucosamine-6-phosphate deaminase was cloned. As this pathway is highly inducible, the regulatory mechanisms have also been studied. Secretion of proteinase by *C. albicans* is also an important virulence factor correlated to adherence and tissue colonization. It has been discovered that the cloned proteinase DNA fragment can be used as species-specific probe to identify *C. albicans* among other species of *Candida*, as conventional tests to identify *C. albicans* employing various morphological, physiological, and biochemical parameters are tedious and time consuming. Recently, the group has isolated the gene encoding a novel transcriptional factor of MAP-kinase signaling pathway which can complement the mating and morphogenetic defect of *Saccharomyces cerevisiae* suggesting a dual function. Moreover, this transcription factor is a strong inducer of pseudohyphae *in S. cerevisiae* haploid and diploids. Prof. Datta for the first time showed the existence of this signaling pathway in *C. albicans* and also succeeded in isolating the MAP kinase of this pathway [49 – 52].

A number of other institutions are also involved with investigations extending from both basic to product and process development aspects in search for solutions and treatment of diseases such as typhoid, tuberculosis, cholera, plague, HIV/AIDS, Japanese encephalitis, malaria, amoebiasis, filaria, leishmaniasis, etc. A few notable activities are described below.

An interactive program on "molecular cloning of HIV type 1 strains in circulation in India" with the major objectives of molecular sequencing of full length *gag* and *env* regions of HIV-1 subtypes is in progress at AIIMS, New Delhi; NICED, Calcutta; NARI, Pune; and CMC, Vellore. Another multicentric project on immunological and virological studies in HIV infection has been implemented at PGIMER, Chandigarh; NICD, Delhi; and AIIMS, New Delhi. The major emphasis of these projects is laid on assessment of cytokine dysregulation in HIV infection, correlation of CD3 number with viral load, and evaluation of soluble markers as predictors of prognosis in HIV infection. Another project on tuberculosis is involved with the collection of *Mycobacterium tuberculosis* strains and clinical samples, evaluation of tests for early detection of *M. tuberculosis* (Mtb), and identification of drug resistant strains of species by molecular probes and characterization of genes associated with the resistance. The primers developed for diagnostic purposes by AIIMS, New Delhi; NII, New Delhi; and CDRI, Lucknow are under evaluation now [2].

IICB, Calcutta, one of the two major CSIR laboratories in biomedical research, has been working on multi-disciplinary investigations of the biology of parasites along with the development of biotechnologies applied to the diagnosis and chemotherapy of *Leishmania* infection. The studies include understanding of the defective homeostasis of the immune network in the disease to provide protection through immunodiagnostics. It has developed an in organello system for studying RNA import. Using this system, ATP-dependent import of a cloned tRNA-Tyr transcript and the role of tubulin antisense binding protein as a membrane bound receptor or carrier for RNA import in *Leishmania* are being understood [53 – 55].

Since the genetic basis of *V. cholerae* tropism and pathogenesis is mostly unknown, IICB, Calcutta has put major stress on comparative genome mapping studies to appraise the extent of genome diversity and in exploring the highly complex questions concerning global regulation of metabolism such as genome rearrangement, control and rate of DNA replication-termination episodes, and the dynamics and evolutionary aspects of genome organization. The reappearance of *V. cholerae* O139 in late 1996, having altered antibiotic sensitivity compared to O139 Bengal, has made intricately difficult the epidemiological scenario *of V. cholerae* and thus necessitated an examination of possible rearrangements in the genome underlying such rapid changes in the phenotypic traits [56]. A separate study on molecular stability and protective efficacy of VA1.3 against clonal types of *V. cholerae* 01 and development of live, oral, recombinant vaccine against *V. cholerae* 0139 has also been initiated for further development of this vaccine at NICED and IICB, Calcutta and IMTECH, Chandigarh.

Malaria eradication continues to remain one of the priority areas of concern. Recent contribution of anti-malarial drug development includes characterization of two complete and one partial gene of *P. vivax* made at AIIMS, New Delhi. One of the genes codes for a unique small heat-shock protein containing the metalloprotease sequence motifs. The other gene codes for a 200 amino acid long polypeptide containing Alu elements. This latter gene is expressed during the erythrocytic phase. This is probably the first malarial protein which contains the Alu elements and is important from the parasite evolution point of view. The third gene isolated is a "pseudogene" for Calcium ATPase. Functional aspects of the gene will have significant bearing on the host-parasite interaction as possible steps towards development of the antimalarial drug [2].

Bharat Immunologicals and Biologicals Corporation Limited (BIBCOL), a new production unit for oral polio vaccine and other immunobiologicals, was established in 1989 in Bulandshahar, based on WHO and US Federal Standards. A products diversification plan has been evolved . So far 33 million doses have been delivered to the National Immunization program. Indo-US Vaccine Action Programme (VAP), a focused initiative for development of new and improved vaccines and diagnostics, under implementation since July 1987, has further been extended till July 2002. The program has led to the identification of two candidate vaccine strains specific to India for rotaviral diarrhea. These strains have been cleared by FDA for limited production for trial in the control of diarrhea in infants and children. The program has also led to the development of two diagnostics assays (PCR & peptide ELISA) for hepatitis C utilizing the Indian isolate of hepatitis C virus [2].

In the area of hormone detection, ELISA kits are being standardized for T3, T4, cortisol, and dehydroepiandrostestrone sulfate (DHES). An ELISA developed by IICB, Calcutta for testosterone is currently being validated by Hormone Research Foundation, New Delhi. Prototype ELISA based kits for detection of estrone glucuronide (EIG), pregnandiol glucuronide (PdG), luteinizing hormone (LH), and follicle stimulating hormone (FSH) have been prepared and are being validated in collaboration with AIIMS, taking care of cross-reactivity of these hormones. Three contraceptive vaccines, namely beta subunit human

chorionic gonadotropin (βhCG) – and anti- riboflavin carrier protein (RCP)-based vaccines for control of female fertility and ovine FSH based vaccine for control of male fertility, are at advanced stages of development at NII, New Delhi and IISc, Bangalore [2].

5 Animal Biotechnology (Including Seri-Biotechnology)

Research efforts in this area include improvement of various techniques of ETT, production of transgenic animals, disease diagnosis, vaccine development, and conservation of indigenous breed of cattle. NDDB Anand, is principally responsible for bringing "white-revolution" in the country through increased productivity through efficient collection of milk from small farmers' cooperatives, processing, and management. It is actively engaged in various R & D projects related to animal health and productivity. Current annual milk production of 70 million tonnes is expected to rise at the present annual rate of growth of 5.5% to 250 million tonnes in 2020 which will be in excess of one-third of global milk output. NDDB has successfully developed vaccines against (a) tropical theileriosis, (b) enterotoxemia in sheep, and (c) a double emulsion against *Haemorrhagic septicaemia*; all these have been transferred to the Indian Immunologicals, Hyderabad for commercialization. NDDB is also actively pursuing a number of projects including: (a) genetic evaluation of crossbred bulls using the concept of Open Nucleus Breeding System; (b) use of genetic markers for selection of economically important traits in cattle and buffaloes; (c) development of PCR- and nucleic acid probe-based diagnosis for brucellosis, tuberculosis and Johne's Disease; and (d) control of parasitic gastroenteritis in ruminants using self-medicating antihelmintic release devices. Bharat Agro-Industrial Foundation, Pune has claimed development of a vaccine and diagnostic kit for IBR disease diagnosis and control. The technology of this vaccine production is shortly being transferred to industry. A major success at National Research Centre for Camel, Bikaner in their research on super-ovulation in camels has been reported. The protocol developed incorporates induction of luteal phase before superovulatory treatment. These R & D efforts have opened up new possibilities for non-seasonal breeding of camels [2].

Buffalo pituitary luteinizing hormone (LH), prolactin (PRL), and follicle stimulating hormone (FSH) have been obtained through biochemical procedures at Delhi University. These preparations contained all the microheterogenous isoforms of the hormones and are ready for distribution for use in ETT. The specificity of polyclonal antibodies of LH and PRL were assessed by immunocytochemical approaches. A cDNA clone of the α-subunit of buffalo FSH has been identified, both as parts of expression vector and of cloning vector for prokaryotic system. The cDNA has been sequenced and shown to be complete and correct. Expression of this clone in competent *E. coli* cells as inclusion bodies has been achieved. National Dairy Research Institute, Karnal, Central Institute for Research on Buffalo, Hissar, and NII, New Delhi are also working on various aspects of ETT including optimization of in vitro fertilization of oocytes, culture of embryos, micromanipulation and embryo cloning using nucleus transfer,

sexing through PCR, reproductive ultrasonography, and endocrine profiles for augmenting fertility [2].

In a project implemented at NII, ovine growth hormone (oGH) was expressed as inclusion bodies using a pQE expression vector. The expressed protein was 10–15% of the total cellular proteins of *E. coli*. The recombinant ovine growth hormone was purified from inclusion bodies and refolded into its native conformation. The refolded recombinant oGH was found to be immunoreactive with RIA/RRA characterized by SDS-PAGE, Western blot, N-terminal amino acid sequence, CD, fluorescence spectra, RIA, and RRA. High cell density cultivation was standardized and found to be enhanced to 100 times. In 16 h, 3.2 g l^{-1} of oGH was produced which is claimed to be the highest level of any recombinant growth hormone reported in *E. coli* [57].

5.1 Seribiotechnology

Traditional rearing of silkworms has been in practice in the country for centuries as silk continues to remain a sought-after commodity from India. The Department of Biotechnology has been supporting a number of projects in mulberry and non-mulberry sericulture. A few important features of such efforts are briefly described below.

A method of microsurgery for the transfer of vector DNA into the developing spermatids of silkworm *Bombyx mori* has been reported by MKU-Madurai suggesting a new line of genetic transformation in silkworm. For correct analysis of gene expression during embryonic development of the silkworm, an improved method to dechlorinate and devitallinize the eggs without damage to the developing embryo has been established at IISc, Bangalore. Similar studies at CSRTI, Mysore, aim to identify DNA markers for the cocoon shell characters of the silkworm breeds and to utilize these RFLP markers as molecular tags to improve traditional silkworm breeding. A technique of molecular characterization of silkworm varieties using micro and mini-satellite DNA has been established at the Seribiotech Research Laboratory, Bangalore. The dendrogram of 13 silkworm genotypes was constructed based on fire microsatellite analysis. The non-diapause and diapause silkworm genotypes have been clearly delineated into separate groups. The microsatellite primers are used to analyze the inbred lines selected for high cocoon and high shell weight and the bulbs generated for resistance and susceptibility to *Bombyx mori* NPV. Fibroin, the major protein of tasar silk, has been isolated and cDNA library constructed from the posterior silk gland of non-mulberry silkworm (*Antheraea mylitta*) at IIT, Kharagpur. Also, polyclonal antibodies have been raised in rabbits against native and denatured forms of fibroin proteins and could be used to screen the cDNA library for cloning the fibroin gene [2].

An immunodiagnostic technique for early detection of microsporidian Nosema infection in silkworms has been developed at MKU, Madurai and transferred to sericulturists [Dharmalingam, personal communication]. Field trials of immunodiagnostic kits developed for detection of the infectious flacherie disease of silkworm *B. mori* are progressing at CSRTI, Mysore. A sandwich

ELISA has been developed at NII, New Delhi by using polyclonal and monoclonal antibody (MA-575), and is being standardized into an immunoassay. A core group has been established at the Centre for DNA Fingerprinting and Diagnostics (CDFD), Hyderabad for the silkworm genome program. The project objective is to construct a high density linkage map based on sequence tagged site markers. Such a high density map would provide all molecular landmarks and signposts that are necessary to clone the genes of interest by reverse genetics, to carry out comparative genomics, to map quantitative trait loci, and to generate inputs necessary for marker assisted selection to make silkworm breeding protocols more efficient and useful. Two diverse silkworm genotypes (Pure Mysore, a non-diapausing genotype and NB-18, a diapausing genotype) have been identified and characterized for the genome program [2].

5.2
Silkworm as a Bioreactor Configuration

Studies are continuing at IISc, Bangalore on the application of *B. mori* nuclear polyhedrosis virus (Bm NPV) to expression of desired genes. Recombinants of BmNPV harbouring *luc* reporter gene under the control of Ac *NPV polh* or p10 promoters gave rise to very high levels of expression in both Bm N cell lines and *B. mori* caterpillars. vBm NPV harbouring the gene encoding hepatitis E-ORF 3, a potential antigen for detection of viral infection is successfully expressed. However, the expression levels were found to be low. Simultaneously, v Bm NPV harbouring the green fluorescent protein (GFP) gene is also generated. Significant expression of GFP is seen in all cell lines which provides a means for the study of virally induced apoptotic phenomenon. Large quantities of GFP are also expressed in the tissues of recombinant virus infected larvae [58–60].

6
Environmental Biotechnology

The ingredients of biotechnology applicable to environmental pollution control and abatement have so far been confined to classical methods of treatment of wastes, both liquid and solid . Elimination of toxic chemicals from the source of their generation has been an important and widely researched area but with little large-scale application concerning domestic wastes or industrial effluent. A few recently introduced studies at NEERI are directed to concern with environmental biotechnology such as:

1. Addition of Mulliplex PCR developing protocols for simultaneous surveillance of enteropathogens in routine post epidemiological monitoring of water quality and organism specific protocols for the detection of *E. coli/Vibrio cholera, Salmonella typhi* and protocols for detection of hydrocarbon (aromatic, halogenated and phenolic compounds in soil samples) utilizing genotypes.
2. *p*-Nitrophenol and *m*-aminophenol utilizing organisms isolated from soil and effluents contaminated with nitro aromatic compounds and continuous culture studies with a view to determine the adaptive transformation of these cells, and their degradative performance.

3. Development of microbial strains for desulfurization of petroleum crude, transformation of sulfur compounds like dibenzothiophene (dbt) exhibiting enhanced specificity, and improved reaction rates on changed nutritional inputs making progress – genes encoding enzymes associated with the transformation of dbt into a fluorescent metabolite are marked in *E. coli* and *Rhodococcus* sp.
4. Development of specific genotypes capable of transforming hydrocarbons in contaminated soil using PCR based techniques – such genotypes are useful in landfill site-selection and characterization. In one project *Pseudomonas putida* has been engineered carrying chromosomal *lux* AB gene encoding *Vibrio harveyi* luciferase beta protein and plasmid encoded cytochrome P450 *can* C gene of the camphor plasmid capable of encoding the monooxygenase only. The constructed cells efficiently dehalogenated hexachloroethane to tetrachloroethane and 1,2,3,4,5,6-hexachloroethane to 3,4,5,6-tetrachlorocyclohexane at rates three to six times higher than that of the natural strains.
5. A two stage redox process for desulfurization of biogas with concomitant recovery of sulfur from the gas consisting chemical oxidation of H_2S in presence of ferric sulfate as H-acceptor followed by microbial oxidation of the ferrous sulfate in which regenerated ferric sulfate is recycled to the first stage has also been reported. NEERI has established a pilot facility yielding 100 m^3 biogas per hour containing 2 – 3 % H_2S. (It is doubtful if (a) the two step process is energetically efficient and (b) the claim of very high (2 – 3 %) content of H_2S in the biogas is true; capital investment in such a four-step operation plant also seems to be very high).
6. Studies on a sequential oxidative reductive process of degradation of chloroaliphatics and chloroaromatics have been under investigation since 1995. Anaerobic breakdown of o-chlorophenol (100 mg l^{-1}) and 2,4-dichlorophenol (100 mg l^{-1}) into catechol acclimatized upflow fixed bed biroeactor is reported to have reached 75 % and 55 % respectively [61, 62].

In a communication Latkar and Chakrabarti [63] reported anaerobic stabilization and near complete removal of urea (< 10 ppm) from an initial load varying between 500 ppm and 1000 ppm in the effluent discharged by an urea plant located in Surat. The treatment is carried out in a continuous 300 m^3 upflow anaerobic sludge blanket reactor having bed volume of 150 m^3. In early 1979 a novel technique of immobilizing viable active cells of *Sarcina ureae* B 84 cells anchored on freshly spun triacetate of cellulose followed by killing the cells in toluene and placing the fibres in an upflow column was developed. The packed column used for instantaneous hydrolysis of urea has been reported [64].

In the Indian scenario two basic components of environmental biotechnology appear to be missing – there are incomplete and unrelated data base situations in which biotechnology development is greatly affected and, under the existing conditions, a big gap exists between the available research-generated data and the methodologies practised. As in most countries, Indian industry's reluctance to invest in pollution control by biotechnological methods is understandable for various reasons. Whereas 25 years of progress in air and water

quality systems in the west is to be applauded, their benefits have not reached most developing countries like India where needs are most striking.

Studies on new biotechnology-based pollution control are not reported in India. A widely studied subject area is anaerobic digestion based on well known techniques. While biogas is an important non-conventional source of rural energy in a country like India where several agencies have put in substantial funds over the past three decades, effective methods of operation and maintenance as well as training of rural people associated with the system can hardly be said to be of much consequence. Above all, good management of the environment requires easily accessible information, documentation, and upgraded technology. Both public officials and citizens, particularly those residents in the vicinity of high pollution sources, may not always be aware of the hazards involved and the possibilities of their abatement via biotechnology routes.

7
Food Biotechnology

Biotechnological interactions in processing food crops and horticulture produce have not been commensurate with the country's needs. It means that, despite excellent facilities and fairly good scientific manpower, very little breakthrough in biotechnology of food processing has been visible. Research has mostly been directed to some classical problems, handling efforts through traditional approaches using mixed cultures and enzymes. A few R & D studies in food biotechnology carried out at the CFTRI, Mysore are mentioned below:

1. In the area of aflatoxin problems, cultures of *Pseudomonas sp*. have been observed to have efficacy to destroy the toxin in concentration up to 200 ppb; similarly, culture filtrates of a strain of *P. aeruginosa* exhibited the ability to completely eliminate aflatoxin (10 μmg to nil) through excretion of a low molecular weight protein acetylcholineesterase immobilized on polymeric membranes by gelatin entrapment [65].
2. Enzyme tannase used in instant tea production has been studied using *Aspergillus. niger* PKL 104 and *Aspergillus niger* MRRS-234 for application as industrial food enzymes. The second strain was found to express very high α-galactosidase, Almost complete recovery of starch from tapioca (cassava) chips without mechanical disintegration has been claimed. Umran variety of Ber (*Ziziphus mawistiana* Lam) has been successfully processed to produce juice from the fruit without deseeding and with no detectable presence of any toxic substance [66].
3. Microbial polysaccharides from *Xanthomonas campestris*, notably xanthan gum for use in food industry, have been studied. Other polysaccharides like dextrans, pullulans, scleroglucan were isolated from several microbial sources. Incorporation of xanthan gum in traditional Indian fermented foods like Idli and Dosa has been investigated in elaborate details. Other products with supplementation of xanthan gum which have been investigated include orange and lemon squash, commercial tomato soup, yogurt preparations with or without CMC. Immunological methods for detection of xanthan gum in

various products were developed and antibodies raised in test animals against xanthan were compared with commercial Kelco samples by dot blot test [67].

4. Xylose isomerase production by biochemical and genetic techniques using *Streptomyces coelicolor* A 3(2) to suit process requirements including desensitization against Ca^{2+} inhibition and rendering it optically active below pH 7.0 and above 60 °C has been achieved [68]. Biosensors/chemosensors for evaluation of food and fermentation products, notably for estimation of glucose and oxygen concentrations in bioprocess fluids, have been developed. The response of the biosensor developed is claimed to correspond to the entire physiological range of glucose over 0.1 – 5 g l^{-1} [19].
5. Work on a few other industrial enzymes of importance in food industry, like proteinase, cellulase, polygalacturonase, and α-amylase has been done. Data on studies of Clarienzyme for use in dehairing are being analyzed in large scale solid state bioprocess in collaboration with Central Leather Research Institute, Chennai [2].

8 Industrial Biotechnology

Bulk products like alcohol, biopesticides, drugs, antibiotics, enzymes, vaccines, some organic molecules, and bioreactors are included in this sector.

As of 1997, India had nearly 250 alcohol distilleries producing annually about 1.3 billion litres of alcohol based on cane molasses, mostly in batch processes with around 4 – 6 wt% ethanol. Significant demonstrations in the development of high ethanol tolerant (< 12 wt% ethanol) osmophillic yeast strain in immobilized biroeactors running under sterile state over many months were extensively reported in the period between the mid-1970s and early 1980s [69, 70]. High ethanol-tolerant strains of yeast have been reported by IMTECH Chandigarh (Ghosh, personal communication) and know-how transferred to three large distilleries in India (Table 2). Downstream products of Vam Organic Chemicals Ltd., Gajraula are based entirely on 300 kl (value Rs. 3 million) of ethanol captively produced daily from cane molasses [Bang, personal communication] From the spent wash of the distillery, 140,000 m^3 of biogas are produced daily whose value as coal equivalent is claimed to be Rs. 210,000. The technology has been imported from M/S Biotin n.v. of Belgium and adapted. Another success in the use of biogas based on digestion of distillery spent wash to generate power has been commissioned at K. M. Sugar Mills Ltd., Faizabad. The unit recently installed with funding from Ministry of Non-conventional Energy Sources claims to generate one megawatt of power using 12,000 m^3 per day of biogas [72]. Cane molasses is also used in the production of nearly 5000 tonnes of citric acid. Several foreign companies have signed MOUs with the Indian partners to increase the existing capacity to nearly 35,000 tonnes per annum.

Nearly 95,000 tons of glucose and dextrose are produced by enzymatic liquefaction of starch, mainly tapioca (cassava). Since lactic acid-based biodegradable polymers like polyglycolide and polylactide are not produced in India, consumption of lactic acid is confined to food processing and the pro-

Table 2. Technology transfers and patent claims in biotechnology [2, 71]

	Know-how & Developer	User
1	Formulation of injectable liposomal Amphoterecin B; KEH Hospital, Mumbai	M/s Ace Laboratories Ltd., New Delhi
2	Western Blot test kit for detection of HIV-I and II injections in blood; Cancer Research Institute, Mumbai	M/s J. Mitra & Company Ltd., New Delhi
3	Agglutination based detection kit for HIV-I & II infection in blood; Univ. of Delhi, South Campus, New Delhi	M/s Cadila Pharmaceutical. Ltd., Ahmedabad
4	Filariasis detection kit; Mahatma Gandhi Institute of Medical Science, Wardha	M/s Cadila Pharmaceutical. Ltd., Ahmedabad
5	Amoebic liver abscess; NII,New Delhi	M/s Cadila Pharmaceutical. Ltd., Ahmedabad
6	Pregnancy DOT-Elisa; NII, New Delhi	M/s Cadila Pharmaceutical. Ltd., Ahmedabad
7	Blood Grouping monoclonals; NII, New Delhi	M/s Cadila Pharmaceutical. Ltd., Ahmedabad
8	Leprosy immunomodulator; NII, New Delhi	M/s Cadila Pharmaceutical. Ltd., Ahmedabad
9	Pregnancy Slide test latex agglutination; NII, New Delhi	Ranbaxy Laboratories Ltd. New Delhi
10	Typhoid fever detection unit; AIIMS, New Delhi	Ranbaxy Laboratories Ltd. New Delhi
11	Pregnancy DOT-ELISA; NII, New Delhi	Ranbaxy Laboratories Ltd. New Delhi
12	Typhoid fever detection kit; NII, New Delhi	M/s Lupin Laboratories, Mumbai
13	Polypeptide P from bitter gourd; Univ. of Rajasthan, Jaipur	M/s Lupin Laboratories, Mumbai
14	Hepatitis B detection kit; NII, New Delhi	M/s Lupin Laboratories, Mumbai
15	Bamboo by tissue culture; Univ. of Delhi, Delhi	Tata Energy Res. Inst., New Delhi
16	Animal birth control injection; NII, New Delhi	Karnataka Antibiotics & Pharmaceutical Ltd., Bangalore
17	Human filarial parasite antigen detection Kit; CDRI, Lucknow	M/S Malladi Drugs & Pharmaceuticals Ltd., Chennai
18	Microbial Conversion of benzaldehyde into L-phenyl acetylcarbinol; CDRI, Lucknow	Atlus Laboratories., Ambala
19	F-MOC derivatives of 12 amino acids, CBT, New Delhi	Atul Products, Balsar
20	Leishmaniasis detection kit; CDRI, Lucknow	Span Diagnostics Ltd., Surat
21	Monoclonals to M13 phage proteins III & VIII; Univ. of Delhi, South Campus, New Delhi	Pharmacia Inc., USA
22	Osmo and high ethanol tolerant genetically modified yeast strains; IMTECH, Chandigarh; Patents applied for: Indian748 Del/93; 749/Del/93 and 1718/Del/94; US Pat 5693526	(i) McDowell & Company (ii) India Glycols Ltd. (iii) Dhampur Sugar Mills

Table 2 (continued)

	Know-how & Developer	User
23	Recombinant oral vaccine against cholera jointly worked out by IMTECH, Chandigarh, IICB Calcutta, and NICED, Calcutta. IMTECH group has filed two Indian Patent applications (2734/Del/96;2740/Del/96) and one European application (EP973099575)	No technology transfer reported yet
24	Plant tissue culture vessel for micro propagationof plantlets including rootlings and shootingof various plant species; IIT, Kharagpur	M/S Tarson Products, Calcutta
25	Enzyme formulation based on cellulase and xylanase enzymes for poultry feed; IIT Delhi.	M/S Tetragon Chemie Ltd., Bangalore
26	*Trichoderma* based biopesticides; IIT Delhi.	Expected to be transferred to industry soon.
27	Biopesticides based on *Bacillus thuringiensis* and Bacillus sphericus; Anna University, Chennai.	M/S Tuticorin Alkali Chemicals & Fertilizers Ltd., Chennai
28	Biodart series of enzymes for leather, detergent and food industry, and Biogold series of microbial fertilizers (*Rhizobium*, *Azospirillum* and Phosphobacteria); Southern Petrochemicals Industries Corporation Ltd., Chennai	Southern Petrochemicals Industries Corporation Ltd., Chennai

duction of pickles, beverages and as food preservatives; a few other uses like textile sizing and pharmaceuticals are limited.

Penicillins along with cephalopsporins are currently the most frequently prescribed antimicrobial agents. The most widely used molecules in these families are semi-synthetic penicillins and cephalosporins derived from 6-amino penicillanic acid (6-APA) and 7-amino deacetoxy cephalosporanic acid (7-ADCA). Out of a global production of 64,000 MMU Pen G and V (approximately 40,000 MT) India processes nearly 16% annually but produces only about 9% in its five units. During the last four decades only four plants, two in the public sector and two in the private sector, were engaged in penicillin production. Three new plants of large capacities, i. e., Torrent Gujarat Biotech. Ltd., Baroda, JK Pharmaceutical Ltd., and Southern Petrochemicals Industrial Corporation (SPIC), both at Cudaloor, have been commissioned since 1995. Amongst the older plants, two have been closed down and one has slowed down its production. The fourth plant at Hindustan Antibiotics Limited (HAL), Pune which has been leased out to Max GB India has enhanced its production of 2200 MMU per annum. At present, against the total installed capacity of over 6500 MMU of Pen G in the country, the production from the five plants during 1997 – 1998 was 5800 MMU. Since the consumption of the domestic market was 5000 MMU, the figures presented an excess production of over 800 MMU and created

a situation of distress selling which resulted in pushing the prices of Pen G and its derivatives to rock bottom levels with the consequence of heavy losses to the producers. The country also has about 50 small semi-synthetic penicillin and cephalosporin producing units. Due to the crash in prices of PenG, about 25 units have already been closed down and another 30 are at the brink of collapse. Thus, although the country is self-sufficient in producing this vital antibiotic and its derivatives, the over-production has created an unprecedented situation for the penicillin industry due to poor planning and irrelevant management. To safeguard the industry, which could have saved around Rs. 2000 million in foreign exchange annually if run properly, the country needs to import these antibiotics at a higher price to take care of the health of the industry and thereby of the people of the country [73]. Despite many debates and seminars, no single research institute alone or in collaboration with any other competent groups of biotechnologists has been assigned this important task of high national priority.

In a communication [71], it is mentioned that R & D spending of the pharmaceutical companies was just over Rs. 1200 million compared to the total turnover of Rs. 90 billion, i. e., about 1.3 %. In-house R & D, particularly in basic research in high risk areas, could never have been in place.

It is good to report that Novo-Nordisk of Denmark has signed a research agreement with a Hyderabad-based Dr. Reddy's Research Foundation and obtained an exclusive world wide license with advance payment to develop and market pharmaceuticals for treatment of obesity and dyslipidaemia based on compounds discovered and patented by the Foundation [74]. Astra Research Laboratory, Bangalore, established by a similar Foundation, has been seeking new knowledge base in the area of molecular drugs. This laboratory is endowed with state-of-the-art infrastructural facilities and several Indian scientists have shifted their place of work from USA and Europe to Astra.

Another Indian biotechnology Company, Biocon India Limited, was founded in 1978 by an innovative entrepreneur as a joint venture with Biocon Biochemicals Ltd. of Ireland. Papain, isinglass (a marine hydrocolloid), and a number of hydrolytic industrial enzymes were the first line of products of the company. Eleven years later, between 1989 and 1997, it rose rapidly and ventured in several new directions with joint affiliations with Unilever in 1989, and set up a 100 % export oriented unit (Biochemizyme India Ltd.) for production and marketing of solid state bioprocess based enzymes. In 1994, Biocon India established Syngene International (P) Ltd., a contract R & D company focused on new drug development using combinatorial and rDNA technology. More recently, the company has gone into further expansion and promoted a new joint venture with Quest International, Holland. Syngene group has recently diversified into pharmaceutical products and formed a new company, Helix Biotech. (P) Ltd. Biocon claims to have been investing 50 % of the profits in R & D and carrying out contract research for a number of biotechnology multinational giants like Glaxo-Welcome, Bristol Myers, Squibb, Astra AB, and Unilever, etc. It also has a marketing tie-up with Genencor of USA (Mazumdar, personal communication).

CDRI, Lucknow reports on a number of successful products in the development of medical, agricultural, and bioproducts. The important ones are

L-ephedrine – nearly 400 tonnes are being produced annually using the process know-how developed by CDRI, and bioinsecticide, a dust formulation substance based on *Bacillus* culture expressing killer larvicide for mosquitoes produced on a 1500 l scale. The formulation has undergone extensive field evaluations and is claimed to be effective on both *Anopheles* and *Culex* species. Further scale-up operations have been tried in collaboration with Hindustan Antibiotics Limited (HAL), Pimpri in 17-kl batches [Behari, personal communication].

Increased emphasis on the application of biological agents for improvement of crop productivity is clearly visible. *Rhizobium* strains specific to various crops such as chickpea, mungbean and soybean have been successfully grown in 100-l capacity bioreactors with high productivities. For control of plant pests, NPV of *H. armigera,* NPV of *S. litura,* GV of *C. infuscatelus, Trichogramma, Chrysopa,* and *Trichoderma* are being commercially produced at two units at Coimbatore and Madurai. Novartis India Ltd. and Wockhardt Ltd are also producing several formulation for control of plant pests [75].

There are a number of examples of "half way through" breakthroughs such as:

1. Cross-linked cellulose based adsorbent beads have been under development at University Department of Chemical Technology (UDCT), Mumbai, and IIT, Delhi for use in expanded bed chromatography. Properties of the beads are claimed to be equivalent or even superior to the imported products. Purification protocols for expanded bed chromatography using cellulose beads for several enzymes are being developed.
2. Osmania University, Hyderabad announced a microbial strain for efficient biosynthesis of *Cephamycin* C.
3. Viswa Bharti University, Shantiniketan has isolated two fish spawning inducing peptides with demonstrated increase of spawning efficiencies in sweet water carp.
4. Bose Institute, Calcutta, jointly with Burn Standard Ltd. has reported solubilizing silica from magnesite ores having more than 6% SiO_2 using a few isolates strain *of Bacillus licheniformis*.
5. IICB Calcutta improved the existing method of leaching of copper from low grade Indian ores using *Acidianus brierbye.* In the area of bioremediation demonstrations conducted by NEERI, Nagpur, for restoration of manganese and coal mine spoils covering a total plantation area of 40 hectares, tests have been successfully conducted by using different blends of industrial effluent and farm yard manure having been inoculated with microbial biomass *of Azotobacter, Rhizobium* and VAM *Mycorrhizae*. Different species of economic plants like, teak, neem, bamboo, cassia, and fruit trees like mango, custard apple, tamarind, etc., were grown in these areas.
6. Specificities of enzymes used in the transformations and modification of azarachtin, salanain, and morphine alkaloids produced by *Mucor* and *Bacillus* spp. are under evaluation for use in the enzymatic synthesis of some narcotic drugs and intermediates at IISc, Bangalore.
7. IISc has also set up a demonstration unit for bioleaching of refractory gold ores and concentrates at Hutti Gold Mines, Karnataka. It is working on the adaptation and improved tolerance of *Thiobacillus ferrroxidans* to *As*–III.

8. CBT, Delhi claims to have made progress in developing a technology of interleukins IL2 and IL10 by recombinant DNA technique; these genes have been subcloned in pGEB-KG plasmid. The two genes were also cloned into pVex115+T vector and positive clones confirmed by restriction enzyme digestion. Three *E. coli* strains were inducted with these clones and proteins expressed with IPTG by growing *E. coli*. Useful technology status of these recombinants has not been reported.

8.1 Intellectual Property Rights in the Biotechnology Sector

India, along with 125 countries at a ministerial meeting held in 1994 in Morocco signed the Final Act of the Uruguay round of multilateral trade negotiations, launched in 1986 through "Declaration" adopted by the member countries. This act was the most ambitious of the trade around under the General Agreement on Tariffs and Trade (GATT) as it covered inter-alia issues related to Intellectual Property Rights (IPR). There are seven areas of IPR which are covered by Trade Related Intellectual Property Rights (TRIPS), namely Trade marks, Trade Secrets, Industrial Designs, Copyrights, Integrated Circuits, Geographical Indication, and Patents. While the Indian laws, regulations, administrative procedures, and judicial system for the first six cases are at par with the rest of the world, in the area of patents the Indian laws are substantially different from the provisions of WTO. As far as biotechnology-related products and activities are concerned, the provision of the WTO differ from those of Indian Patents System in a number of major areas such as:

- WTO provides "Product patents" in all branches of technology while the Indian Patents System does not provide "Product Patent" in drugs, food, and chemicals excepting only "Process Patents."
- WTO grants patents for any inventions (products or processes) in all fields of technology provided they are *new*, involve an *inventive step* (non-obvious), and are capable of *industrial applications* (useful), but provide flexibility for exclusion from patentability in areas like (i) diagnostic, therapeutic and surgical methods for the treatment of humans and animals, (ii) plants, (iii) animals, and (iv) essentially biological processes for the production of plants or animals. WTO, however, provides patents on microorganisms and microbiological processes. In contrast, Indian patents laws do not allow patenting of any life form, although, patents based on microbial processes are permitted.
- WTO requires protection of plant varieties either by patents or by an effective "sui generis" system or by any combination thereof, while at present there is no system for protection of plant varieties in India.
- WTO provides 20-year uniform duration for coverage of patent life for all patents while the Indian System provides 7 years for food and pharmaceuticals and 14 years for others.

While India has yet to decide on the patentability of living forms, what is being considered on the subject is that expression hosts as microorganisms designed by human intervention through the process of genetic manipulation which are

not available in nature and the design of which is non-obvious, would be patentable under WTO. The country is at present debating on the need to define and categorize the patentable microorganisms required as per the WTO. Currently the entire subject is before the national parliament.

It is important to mention here that by joining WTO, India is expected to receive "Most Favored Nations Treatment" from all other member countries and therefore there would neither be any special need to enter into the bilateral collaborations with any member country for international trade nor any possibility of a member country imposing special sanctions to destabilize India's international trade. It is envisaged that conventional biotech industries would continue to grow well in India and WTO will have essentially no deterrent effect on this process. It is, however, feared that health care products produced by the use of efficient microbial processes, genetic recombination, and hybridoma technology will be very expensive in India after the enactment of WTO.

Technologies are becoming increasingly R & D driven. R & D investments create intellectual properties which are to be protected, and the WTO system which recognizes such needs by rewarding and encouraging innovations has been accepted by all the Member Countries. In exceptional scenarios, to promote and adequately protect public interest, India is free to adopt measures consistent with WTO to direct authorization of use of such patents which may adversely hinder the causes of public ethics and interest. In the emerging situation, following the enactment of WTO, R & D base of the industry is likely to be strengthened and greater collaboration between industry and R & D Institutions is foreseen, especially for developing competitive biotechnology processes [76–78].

9 Conclusion

Indian efforts that went into biotechnology development in the course of the last two decades have been scanned on the basis of information available from sources engaged in the field. The strongest inputs include location of infrastructure in a number of institutions and universities, building a few centres of excellence, and support to existing academic set up already well known world-wide. Emphasis has been laid on research areas considered appropriate to seeking solutions for many problems of health care, agriculture, and animal productivity.

Materials incorporated have been drawn mainly from two sources: (a) Annual Reports published by a number of research institutes and Government agencies; and (b) brief but useful write-ups received on request from colleagues heading major biotechnological activities in the country. All reports incorporating significant important study-based data and results on what constitutes India's important activities in biotechnology have been presented. Out of 22 centres of biotechnology activities contacted, 14 responded, a few promised, and the rest kept silent. The other difficulty was to figure out how much of the reported material could qualify incorporation in the paper. This being the nature of the prevailing situation, what is modestly possible has been done.

While the estimated Indian biotechnology market has been placed at Rupees 15 billion (~ $ 350 million) as of 1994 of which one third pertains to

pharmaceuticals, one-fifth to horticulture and vaccines together, one-tenth to diagnostics and instruments combined, only 2% to plant tissue and fluoriculture and more than one-third for all the rest together [79]. The review reveals that the country is far from reaching the target due to many factors. A combination of world class facilities, a fairly good scientific workforce engaged in R & D, and strong political recognition of the importance of biotechnology in academia and industry failed to make any quantum jump to gain a position amongst the top ten in the world. The chemical industry's reluctance to make substantial investment in R&D, both in- house and in academia, is very much in evidence. Moreover, public awareness of the benefits and evils of biotechnology is largely missing.

Except for a few diagnostic kits, antibiotics, enzymes, and agricultural and horticultural improvements, a real blockbuster Indian product has not yet appeared on the market. Good Indian sciences in print comes quickly to the knowledge of peer colleagues and scientists are rewarded with positions and awards. However, there are intractable difficulties such as (a) to produce and sell a marketable bioproduct/bioprocess massive investments, (c) to retain world class bioscientists and engineers in mission mode projects, and (d) to make reliable assessment of what can be done with quality. Realization of these problems is slow but in place. Often public announcements are loud and blown up, leading to complacency and elated self image. Based on the factual position given in the paper, the country will take several more years to leapfrog to a few targets. However strength lies in excellent infrastructure facilities in several centres, qualified human resources, competitive research costs in global terms, a vast biodiversity of resources, large market size, and the possibility of tapping the knowledge-base of non-resident Indians. The most important weakness lies in the bioproduct development related factors like abundance of non-focused research, low industry spending on R & D, many national laboratories' research being targeted to academic degrees, ineffective R & D management in public funded institutions, and both politicians' and bureaucrats' inability to accept these facts.

And yet another truth lies elsewhere. The quality of scientific contributions currently made in new biology and biotechnology at a number of world class academic and research centres, as the review highlights, leads to a buildup of confidence. Two decades of research in a new science and technology in a developing country burdened with innumerable problems is certainly not enough time to make news-breaking announcements other than through the wishful thinking of politicians. Much has been expected though. Therefore, given the current biotechnology status, one need neither be pessimistic nor unduly complacent about the chances of immediate major gains in biotechnology in India. The education and training programs have been well laid, intelligent scientific work-force being available, fairly good infrastructures are in place and industry is gradually becoming aware of the fact that the taxpayer can only support the business of R & D in biotechnology up to a point, and beyond this it must be the responsibility of the industrial sector for its survival in the face of global competition already clearly visible. It is hoped that the nature of resilience of this ancient nation will produce a turn-around in the early decades of the new millennium.

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History of Biotechnology in Austria

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Austria has contributed significantly to the progress of the biotechnologies in the past and is actively engaged in doing so today. This review describes the early history of biotechnology in Austria, beginning with the Vienna process of baker's yeast manufacture in 1846, up to the achievements of the 20th century, e.g. the submerged vinegar process, penicillin V, immune biotechnology, biomass as a renewable source of fermentation products (power alcohol, biogas, organic acids etc.), biopulping, biopolymers, biocatalysis, mammalian cell technology, nanotechnology of bacterial surface layers, and environmental biotechnology.

Keywords. Early history of biotechnology in Austria, Vienna process for baker's yeast production, Submerged vinegar fermentation, Penicillin V, Cell culture, Human plasma and immune biotechnology, Biopulping and lignocellulose conversion, Bioprocess technology, Environmental biotechnology, Genetic engineering

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1 Introduction

Biotechnology, if it can be considered a trade, can be traced back many centuries, when wine making, brewing, production of vinegar and distilling were important human skills. The history of biotechnology as an industry apparently begins in the early 19th century, parallel to the gradual general change in industrialization in Europe and America.

Austria, i.e. the country now represented by the Republic of Austria, has contributed considerably to the development and progress of biotechnology. The beginning of this remarkable history may be traced back to the first decades of the 19th century although in this country earlier flourishing trades, such as wine making, brewing, distilling and the production of vinegar, were also practiced for many centuries.

In 1815, the Vienna Polytechnic Institute (Fig. 1), now the Vienna University of Technology, was founded. From the very beginning biotechnological subjects were taught. The founder and first director of the Vienna Polytechnic Institute, Johann Josef Ritter von Prechtl (1778 – 1854), was the author of a renowned textbook of chemistry with special reference to chemical technology (1813) and,

Fig. 1. The Vienna Polytechnic Institute near St. Charles Church

together with Altmütter and Karmarsch, was the editor of a 24-volume "Technological Encyclopedia or Alphabetical Handbook of Technology, Technical Chemistry and Mechanical Engineering" (1830 ff). Teaching and research at this institute contributed considerably to the progress of Austrian industry at this time.

2 The Vienna Process for Producing Baker's Yeast

An early example of Austria's historical role in biotechnology was the development of this process to produce baker's yeast. Until the 19th century, bakers obtained dough-leavening yeast mainly from local breweries which produced beer by the so-called top fermentation, where the yeast was recovered by skimming off the foam and separating the yeast mass by settling and sieving. When brewers changed to the more efficient bottom or lager fermentation, the resulting bottom yeast was inferior in quality and in quantity of supply. For example, in Vienna, the capital of the Austrian Empire, more than two hundred bakers seriously complained about this shortage. Distillers, although producing alcohol by a similar process using top yeast, were unable to suffice the increasing demand. Therefore, in 1847, the Federation of Industry of Lower Austria decided to offer a reward of 1000 gulden together with a medal worth 50 ducats to the person who could produce an amount of 22.4 kg of yeast plus 40.74 L of alcohol from 193.8 kg of grain (values calculated from measures of that time). A further

condition was that the competitor must prove his ability to supply and sell an amount of at least 5000 kg of this yeast during a period of one year at normal market price.

The competition was won by Julius Reininghaus, a German chemist who had learned the Dutch art of yeast manufacture in Hannover and had offered his services to Adolf Ignaz Mautner, the owner of a brewing and distilling establishment in Vienna [1]. Reininghaus was able to obtain yields even exceeding the requirements of the competition. Furthermore, he successfully introduced maize as a raw material for yeast production. He became Mautner's partner – and his brother Johann Peter became Mautner's son-in-law! Several additional production companies were founded and at the present time these two family names still represent renowned Austrian establishments. It was only about 70 years later that the Vienna Process was replaced by the more modern procedures involving aeration and feeding of the carbon sources (Zulaufverfahren).

3
Technical Mycology, a Novel Field

Winemaking, brewing, distilling and the production of vinegar were already being taught at the Vienna Polytechnic Institute in the schedule of the school of special technical chemistry in 1816. Beginning with the work of Louis Pasteur, who established the scientific essence of these trades by studying and proving the biological and biochemical nature of fermentations, these fields developed into large industries with enormous production figures. Following the foundation of various research institutes, such as the Institut Pasteur in Paris, the Institute of

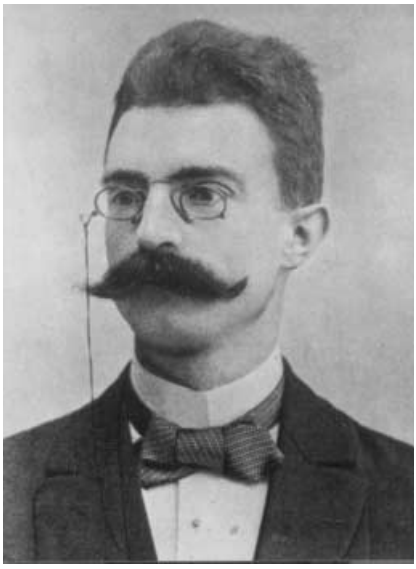


Fig. 2. Franz Lafar (1865–1943), the founder of Technical Mycology

Fermentation Research in Copenhagen and in Berlin, Austria also decided to establish a special university institute. This institute was founded at the Vienna Technical Institute in 1897 and still exists as the Institute of Biochemical Technology and Microbiology at the Vienna University of Technology. Its first director and professor was Franz Lafar (1865 – 1943) from Vienna (Fig. 2).

Lafar had worked at the Agricultural Institute of Hohenheim and as a lecturer at the Stuttgart Technical Institute. He had gained considerable reputation as the author of the two-volume "Handbook of Technical Mycology" in 1896 (English translation, 1898; Russian translation, 1903). This was followed by a five-volume second edition (1904 – 1914) which became a standard source of a novel discipline, Technical Mycology, a designation that he himself coined. Soon after, Technical Mycology was also taught at the Graz Technical Institute [2].

4 Improvements in Distillery Practice

Besides his fame as one of the pioneers of the new field, Lafar also earned acclaim for the improvements he made in distillery practice. Distillers originally produced alcohol by purely empirical methods, using grain or potatoes as raw materials and the natural yeast flora within the distillery. Later, yeast was collected from the first batches of a production and used to seed successive batches, and this was carried out throughout the production campaign. Accordingly, severe contaminations were encountered. Through the work of the Berlin Institute (Delbrueck), pure culture yeast ("Kunsthefe") became available and it was especially recommended that this "artificial" yeast be propagated under conditions of "natural pure culture", i.e. adapted to the conditions of the substrates being processed in the respective distilleries.

In order to counteract contamination, mainly from butyric acid bacteria, it was common practice to maintain a spontaneous lactic acid fermentation, which was introduced by the natural bacterial flora of the mash and the environment, and it was hoped that this would remain active throughout the season. In 1893, in an attempt to create optimum conditions for this protective fermentation, Lafar isolated the most potent bacterial strain from an actively souring yeast seed culture and introduced this culture successfully to all the distilleries in the Hohenheim area during the following campaigns. In 1896, after this method had been adopted in the whole Württemberg area, he published his findings [3] designating the organism as *Bacillus acidificans longissimus*, but only mentioned to provide a more accurate description. At the same time, and in the same journal following Lafar's paper, Leichmann [4] described the isolation of a similar strain, which he designated *Bacillus delbruecki*, and this was the name to subsist for the apparently identical strain. The designation *Bac. acidificans* (*Bac. delbruecki*) was used by distillers for some time, but nowadays the literature only mentions *Lactobacillus delbrueckii*, in particular, as the organism of the current industrial lactic acid fermentation process.

5
The Advent of Plant Cell Culture

Since plant tissue culture has become a potential biotechnological field, it is justified to investigate the past of this valuable tool. As early as 1839, Schwann suggested that plant cells should be considered totipotent. This means that each living cell of plant tissue is able to develop into a whole organism provided the cell is maintained in a proper environment, esp. with respect to nutrition.

The first experiments with fragmented plant tissues resulting in the formation of actively multiplying cells were performed before the turn of the 20th century. The Austrian scientist Rechinger (1893) even tried to determine and to define the 'limits of divisibility' of various plant materials. It was the great Austrian biologist Gottlieb Haberlandt, however, who in 1902 established the foundations of plant tissue culture [5]. Unlike Rechinger, Haberlandt believed that it was even possible to propagate isolated plant cells. Although his experiments were of limited success, his merit as the founder of this discipline has been fully acknowledged during this century (see, e.g. Krikorian and Bequam, 1969) [6] and quite recently, in 1998, this fact was celebrated in an international symposium.

By choosing more suitable plant material, root tips, and better nutrient media, excellent results were achieved – first by Gautheret in 1934. Since then, plant cell culture has become a fruitful discipline within biotechnology, with manifold economic potential. This includes the production of various products of secondary metabolism as well as e.g. transgenic crops.

Obviously, the photosynthetic potential of plants with respect to the production of biomass as a renewable resource in sustainable production cycles has found actual attention and has been defined in many recent national and international research programs. A special variant of such endeavors has been formulated as "New Phytotechnology" by the Austrian group of Othmar Ruthner and coworkers [7] and this will be dealt with in the following section.

6
New Phytotechnology

The basic idea may be defined as attempts to utilize light (solar) energy in a controlled artificial environment by establishing some kind of plant factory enabling continuous production of any kind of plant independent of site and season. This may be realized on a large (industrial) scale by a three-dimensional driven conveyor system in a closed environment illuminated by a fixed light-lattice. The environmental conditions in such systems (Fig. 3) may be optimized according to the specific requirements of the crop to be produced. Continuous industrial plant production may serve not only to provide fresh vegetables, green fodder, and various plant material for pharmaceutical purposes (e.g. *Digitalis lanata*), but also for the propagation of seedlings or shoots for mass cultivation, e.g. for short rotation forestry to produce renewable energy resources.

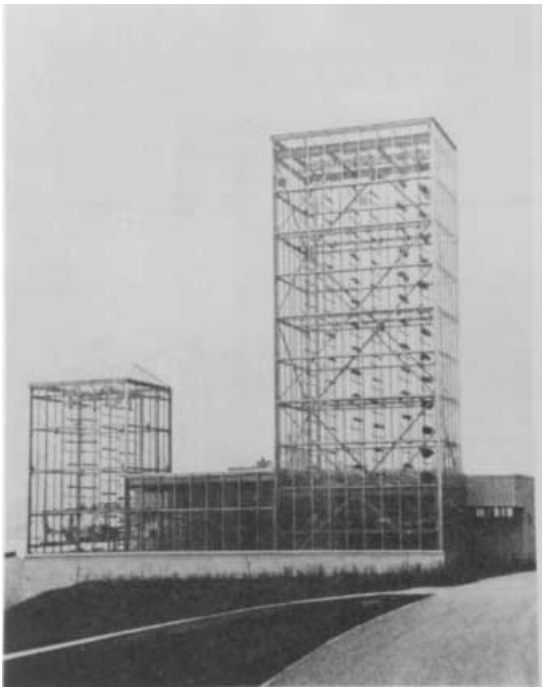


Fig. 3. Continuous industrial plant production system (O. Ruthner)

It has been claimed by the producers of these systems (Ruthner Pflanzentechnik Ltd. and Maschinenfabrik Andritz Ltd.) that, for example, the water requirements in such facilities are only 2% of that in conventional European fodder production. Fertilizer requirements are much lower than in conventional economies and the pesticide demand is reduced considerably. This would suggest its application not only in arid zones but also in space [7].

It should be noted at this point that historically the idea of systematically investigating plants as sources of various raw materials goes back to the great Austrian scientist Julius von Wiesner (1838–1916), who established the science of natural materials (Rohstofflehre) with his famous book, "Die Rohstoffe des Pflanzenreiches", in 1873. Haberlandt was one of his students.

7
An Important Role in Citric Acid Fermentation

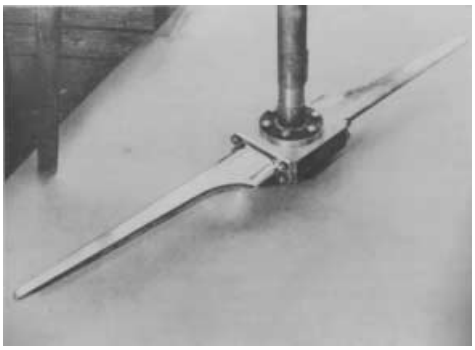
Commercial citric acid fermentation began with the pioneering work of Currie (1917) in the United States, who initiated the first successful industrial production of citric acid in 1923 with Chas. Pfizer in Brooklyn [8]. This venture almost demolished the market position of citric acid from citrus fruits held by Italy. Soon after, attempts were made to establish respective plants in Europe. Interestingly, the first patent was applied for in Austria in 1923 by J. Szücs from

Vienna and granted in 1925 [9]. Szücs offered his knowledge to a company in Prague [Montan- und Industrialwerke, vormals Joh. Dav. Starck (1924)]. As early as 1928, a plant was built at Kaznéjow near Plzen, and this plant went into production using for the first time molasses as raw material, according to Szücs's patents. It was in this plant that the treatment of molasses with hexacyanoferrate was invented [10], a method still in use in industries using less pure raw materials, and which has been studied intensively for decades by several research groups (for reviews see e. g. [11, 12]). Today, Austria is one of the most prominent producers of citric acid in the world.

8
Further Improvements in Yeast Production

About one hundred years after the invention of the Viennese process for baker's yeast production, several improvements to this art were again made in Vienna. W. Vogelbusch, a process engineer and owner of a consulting firm working with Hefefabriken Mautner Markhof, invented several rotating aeration devices to replace the conventional static aerators in baker's yeast production [13, 14]. It had been known since the basic investigations of Pasteur that oxygen suppresses fermentation (Pasteur effect), and this had given rise to the so-called "Zulauf" processes as a new technology of yeast manufacture, comprising low feed rates of the carbon source together with high aeration rates.

The new rotating aerators of Vogelbusch, especially the so-called "dispergator" (Fig. 4a, b) provided higher oxygen transfer rates, thus saving air and enabling higher feed rates of the carbon sources resulting in higher productivities. These feed rates, in turn, were usually adjusted according to empirical schedules owing to the logarithmic law of yeast growth. An attempt was made to keep the con-



a

b

Fig. 4a, b. **a** Vogelbusch dispergator (courtesy of Aktiengesellschaft Kühnle, Kopp and Kausch, Frankenthal, Germany); **b** Vogelbusch dispergator with cooling device and baffles (courtesy of Vogelbusch GmbH, Vienna)

centration of the carbon source as low as possible to avoid excessive aerobic fermentation producing alcohol which would get lost via the exhaust air.

This was the starting point for a further improvement in the regulation of the carbon source feed rate. By measuring the ethanol content of the exhaust air (representing the ethanol concentration in the mash according to Henry's law), using catalytic oxidation of the ethanol and converting the heat generation into an electrical signal, the feed rate could be adjusted elegantly to the oxygen demand, i. e. the oxygen transfer property of the aerator. The so-called "Autoxymax" principle of Vereinigte Hefefabriken Mautner Markhof is in use in many yeast plants all over the world. The initial exhaust gas sensor has now been replaced by a system derived from common smoke detection devices (cf. [15]).

Yet another improvement was of great influence on the economics of yeast production: The separation of the yeast from the spent mash was performed by centrifugation and subsequent dehydration of the resulting yeast cream in a frame press. Only the application of frame presses allowed dry substance values of about 27 % to be attained, this being the desired standard with respect to handling properties and shelf-life. Attempts to replace frame presses with rotating drum filters showed that such dry substance values were barely achievable. The problem was solved in an ingenious way by K. v. Rokitansky and E. Küstler.

Rokitansky, one of the chief chemists in the above-mentioned establishment, had studied not only chemistry but also botany with the famous botanist F. Weber at Graz University. As many readers know, one of the favorite objects of introductory microscopic courses is the onion cell (*Allium cepa*), where in particular the phenomena of cell turgor and cytorrhysis can be studied. When, years after this, Rokitansky was reasoning about the negative results with a rotating drum filter to separate yeast suspensions, he remembered his observations with cytorrhysis experiments, demonstrating the dehydrating action of e. g. salt gradients on cells. Together with Küstler, he developed a method of dehydrating yeast creams on a rotating drum filter by pretreating the yeast cream with a sodium chloride solution and subsequently separating the dehydrated yeast cells on the filter. Adhering salt solution could be removed by quickly spraying with water in a subsequent zone of the filter thus avoiding rehydration of the cells [16 – 18]. With this invention, dry substance values exceeding 30 % could be achieved, which facilitated subsequent adjustment of particular dry substance values and enabled yeast to be provided with improved shelf-life.

Together with a process of combined yeast and ethanol production, the so-called KOMAX process, in which the propagation of yeast is performed in a way that a definable amount of yeast from the ethanol producing stage can be used as seed-yeast for the successive baker's yeast stage, the inventions mentioned above constitute most of the advanced technology of yeast manufacture today which, at least in part, is applied in many countries.

9
Ergot Alkaloids

Brief mention should be made of Austria's part in the history of producing these substances. Through the centuries, ergot alkaloids were the causative agents of

severe epidemic diseases, ergotism. Typical manifestations were convulsive and gangrenous ergotism, and these were handed down under various names due to their striking actions, e.g. *ignis sacer* (holy fire) or *plaga ignis* or *pestilens ille morbus*, etc. (cf. [19]). It appears that the beneficial actions of ergot alkaloids, namely to enhance muscle contractions, esp. to provoke uterus contractions during childbirth, were utilized even before the details of ergotism were known.

Ergot alkaloids are formed by all known (about 50) species of the fungus *Claviceps* and, to a lesser extent, also by some other fungi, e.g. *Aspergillus* and *Penicillium*. *Claviceps* infects mainly grasses, of which rye and other cereals appear as typical examples being responsible for the former epidemic outbreaks of ergotism mentioned above. For medical uses the sclerotia of the fungus were collected from these cereals, especially in rye fields, and processed in small pharmaceutical establishments. The first clinically used compound, ergotamin, was discovered by Stoll in 1918. Obviously, there was increasing interest in developing more productive and controllable methods of production, especially since it became apparent that yield as well as type of alkaloid or alkaloid group was rather strain-specific and dependent on environmental conditions.

This was the beginning of the so-called parasitic production of ergot alkaloids, which was developed in Hungary (von Békésy, 1935 [20]) and improved in Austria (Hecht, 1944 [21]) and Switzerland (Stoll and Brack, 1944 [22]). The essence of these methods was to inoculate ears of rye before or at the time of flowering with a conidia suspension of *Claviceps* by an injection device causing small lesions, e.g. using inverted sewing needles with the ears of the needles as a suitable reservoir for the necessary amount of suspended conidia for infection. Yields per acre of ergot alkaloids could be increased considerably and uniform alkaloid moieties could be obtained.

Today, this method has been replaced by fermentation processes, enabling the production of a wide spectrum of specific compounds by the most suitable strains under the most precise production schedules.

10
The Submerged Vinegar Process

Shortly after the Second World War, in a period of many changes in the economic situation in Austria, two chemists met by chance in an office in Upper Austria, one of which, Heinrich Ebner, was working in a vinegar plant, whereas the other, Otto Hromatka, an organic chemist with a strong pharmaceutical background, was in search of a new field of activity. Reasoning about the fact that vinegar was not produced by a submerged process, the two scientists decided to try to transfer the old-fashioned trickling process into a modern submerged fermentation technology.

The essence of the trickling process (generator process) is to charge a reactor, filled with e.g. wood shavings with an adhering active population of acetic acid bacteria, from the top with wine or beer or diluted ethanol containing a certain amount of vinegar (in order to avoid overoxidation) while aerating from the bottom. In the old Schuezenbach process, vinegar was produced in one step and withdrawn at the bottom. In the more modern generator process with higher

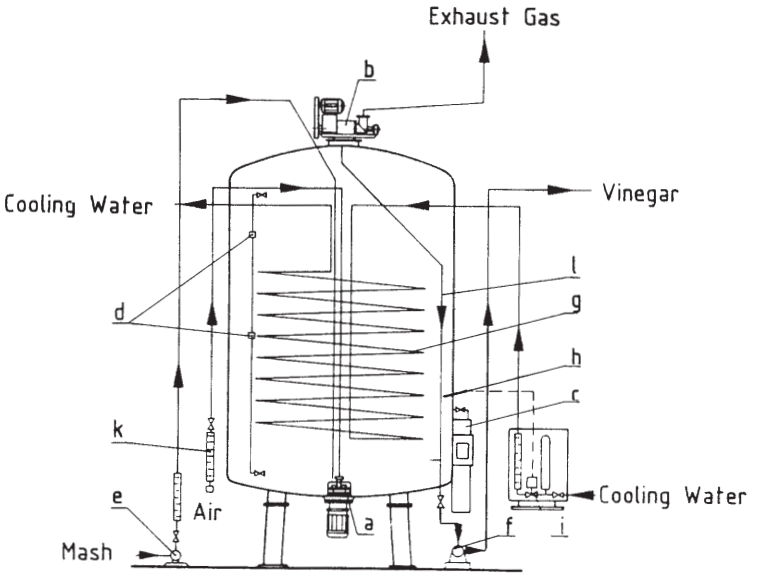


Fig. 5. Modern acetator for the production of vinegar (courtesy of Frings, Bonn)

reactor volumes causing internal overheating, the necessity of cooling required shorter residence times. This was accomplished by circulating the mash and cooling it outside the reactor.

Hromatka and Ebner observed that active acetic acid bacteria in a submerged system were extremely sensitive to interruptions in the aeration. They found that an actively oxidizing bacterial population could not be obtained by the usual procedures of inoculating with a normal bacterial pure culture, e.g. from an agar medium. This could be achieved, however, by placing wood shavings from a working generator into a continuously aerated mash until a certain number of cells became suspended in the mash and began to multiply. In this way, the submerged vinegar process was developed [23, 24]. Subsequently, the know-how was merged with that of Frings Ltd., Bonn, the company now producing this type of vinegar plant.

Present reactors, so-called acetators (Fig. 5), are equipped with self-priming aerators (guarded by an emergency power station), an efficient cooling system and analyzers to determine the composition of the mash in situ. The advantages, as compared with the preceding generators, are much higher productivities, the possibility of producing purer vinegar (acetic acid), e.g. from pure ethanol, and no transient batches when changing the raw material. The only disadvantage is the fact that clarification of the resulting vinegar is more expensive. A great number of plants all over the world have changed to this efficient process.

11
The Penicillin V Story

The discovery of penicillin by Alexander Fleming and its large-scale production, realized by the famous Oxford group of scientists and a consortium of US companies during World War II, has changed our life expectancy almost unbelievably. No wonder that the story of this great discovery has been told many times (e.g. [25]). In contrast, the story of the discovery of the first acid-stable, oral penicillin is less well known – in some of the various sources it is even neglected.

One of the few disadvantages of the common penicillin, designated penicillin G, was its lability under acidic conditions. Therefore, penicillin G could not be administered orally. Moreover, it was difficult to build up stable blood levels because, parallel to its low toxicity, penicillin was excreted within a few hours after injection inevitably demanding frequent treatment. It was therefore acclaimed as a considerable achievement when the desired oral penicillin was discovered.

Soon after the end of World War II, a small plant was established in the Tyrol, then part of the French occupied zone of Austria, in a closed-down brewery of the Austrian Brewing Corporation: the Biochemie Kundl GmbH. Research in this establishment was entrusted to Richard Brunner (later professor at the Vienna University of Technology), who had witnessed the first experiments of a German research group under K. Bernhauer in Prague to produce penicillins during World War II.

Due to the special situation in the post-war era, the implementation of this endeavor was extremely difficult. With the help of a French chemist, Captain Rambaud, of the French occupation forces, a small team of scientists and engineers succeeded in producing sufficiently pure penicillin within a rather short period of time (1948). Problems of equipment were solved by using various redundant military materials, e.g. V2 missile containers as liquid vessels, self-produced fermenters stirred with the help of motors of submarines and aerated by compressors powered by motors of German Tiger tanks. The necessary pipes were obtained from a bombed Innsbruck café. Since corn-steep liquor was not available, yeast extract had to be used, and whey had to serve as a substitute for lactose. Even the necessary butanol for the preparation of the extractant had to be produced by installing a butanol fermentation.

Obviously, one of the major obstacles was the frequent occurrence of microbial contaminations during fermentations which destroyed many valuable batches. In the endeavor to counteract such contaminations, Ernst Brandl (Fig. 6), working on his dissertation in the microbiological and fermentation laboratory, tried to add 2-phenoxyethanol, a compound mainly in use as a preservative in cosmetic preparations, to the fermentation medium. The surprising effect was a significant discrepancy between the results of bioassays and those of chemical (iodometric) determinations in the resultant fermentation broth. This phenomenon was studied by Hans Margreiter (Fig. 6), working with Brunner in the chemical research laboratory of the plant. Surprisingly, when trying to isolate the penicillin moiety by extraction with diisopropyl ether, he observed that crystalline precipitates with penicillin activity had been formed in the acid aqueous phase after prolonged standing. Soon it was realized that a novel, acid-stable penicillin

Fig. 6. E. Brandl and H. Margreiter (courtesy of Biochemie Kundl GmbH, Kundl, Tyrol)

had been discovered, apparently due to the ability of the fungus to utilize the added phenoxyethanol as side-chain precursor after its oxidation to phenoxyacetic acid. The respective patent application was filed in 1952 [26].

The possibility of oral administration of this novel penicillin, designated as penicillin V (phenoxymethyl penicillin), together with its low toxicity, paved the way to high dosage therapy, which was successfully introduced by K. H. Spitzy in Vienna.

Considerable disappointment arose when it was found that the formation of phenoxymethyl penicillin had already been described, and patented, by O. Behrens from E. Lilly in the US, who had, however, not recognized its acid stability. Successive negotiations were able to settle this problem in a most satisfactory way for both sides and both companies were able to acquire leading positions as producers of oral penicillin. Biochemie Kundl GmbH., now part of Novartis Ltd., is still one of Austria's biotechnical companies most renowned for its production of antibiotics, enzymes and other specialties such as e. g. cyclosporin. Quite recently (1998), the company acquired the fermentation facilities of Hoechst Marion Roussel at Frankfurt to enlarge its production capacity about twofold.

About one third of the world production of oral penicillin comes from Austria.

12 Immune Biotechnology

In 1890, E. von Behring and S. Kitasato published their famous paper, "Über das Zustandekommen der Diphtherie-Immunität und der Tetanus-Immunität bei Thieren". This paper opened the way to the new field of serotherapy, i. e. passive immunization. Soon after, in 1892, the first diphtheria antiserum was produced industrially (Farbwerke vorm. Meister Lucius & Brüning, Hoechst) and, in 1898,

von Behring founded a private research institute in Marburg, known now as Behring-Werke.

In Austria, the "Serotherapeutical Institute" was founded under the direction of R. Paltauf in 1894 to provide the necessary vaccines against diphtheria and tetanus. In their first research activities in this establishment, E. Loewenstein and M. Eisler von Terramare [27] discovered that the toxicity of tetanus toxin could be eliminated when treated with formaldehyde, thus enabling passive and active immunization.

After World War II, one of the first challenges was the fight against poliomyelitis. Having this in mind, a new production facility was founded in the basement of an institute of the Vienna University, the 'Institute of Hemoderivatives'. The founders were H. Eibl, W. Auerswald and O. Schwarz. Eibl had worked with Eisler von Terramare in the field of serotherapy and had successfully produced a poliomyelitis antibody concentrate from human serum by isolating and concentrating the respective immunoglobulin. Although yields were low, the young establishment was able to acquire the Establishment License of the US Department of Health, Education and Welfare.

Besides the production of polio and tetanus immunoglobulins, the fast growing company concentrated on the fractionation of human plasma on a larger scale. To this end, plasmapheresis was introduced as early as 1963 to increase the supply of human plasma. The application of Cohn's method of cold ethanol fractionation gradually enabled the production of a variety of common preparations, but also e.g. coagulation factor concentrates (factor VII, VIII and IX). Today the company processes about 10% of the world supply of human plasma.

During the 1970s, together with the Institute of Hygiene of Vienna University, the development and production of a vaccine against tick-borne encephalitis from chicken embryonic cells was successfully implemented.

In 1975, research on the coagulating properties of blood fractions led to the development of a so-called fibrin tissue sealant, which is active in quick wound closing and successive healing and can be used in many fields of modern surgery.

Monoclonal antibody techniques were applied to produce a concentrate of antithrombin III, which is used in the treatment of inborn thrombic disorders.

Operating since 1960 under the name 'Immuno Ltd.', the company was recently (1996) been taken over by Baxter International.

The acquired immune deficiency syndrome (AIDS) marks another challenge in immune research. In Austria, an indirect AIDS-immunofluorescence assay has been developed by Waldheim Pharmaceutical Co. It has been approved by the US Food and Drug Administration since 1992 and is mainly recommended as a confirmatory test.

So far, the present article has attempted to outline the main historical roots of Austria's role in the development of biotechnology. Needless to say that Austrian scientists and technologists have tried to keep up with the modern biotechnologies, e.g. the applications of molecular biology and gene science for industrial applications, or at least the application of the respective methodology in the study of more practical problems. Space does not allow a description of all the more

recent developments. A tremendous amount of basic research has also been carried out at Austrian universities and research institutes that cannot be covered in this publication. The following section, therefore, should give some impressions of the mainstreams of biotechnological research and development in Austria's industrial and academic institutions during the last decades. The reader may find additional information e.g. in an extensive review by C. P. Kubicek [28] from the author's institute, which covers publications over the period of about 1990 to 1995. Besides this, most of the research institutions in Austria (cf. [29]) and the larger companies (especially pharmaceutical ones) provide material on the Internet.

13
Renewable Resources for the Supply of Energy and Chemicals – Biomass

The fact that Austria decided to do without atomic energy, and the oil crisis of 1973, greatly influenced the country's attitude in favor of alternative energy sources. Several programs involving industry and university institutes, supported by the Austrian government, were thus initiated in the following fields:

- Production of power alcohol from indigenous raw materials,
- Production of biogas, i. e. methane, from waste materials,
- Re-exploration of the potential of cellulosic and lignocellulosic materials, and
- Reinvestigation of the acetone-butanol-ethanol (abe) fermentation.

In these programs, various university groups (Vienna and Graz University of Technology, Graz and Linz University, Vienna University of Agriculture), together with research laboratories from industry, have conducted research on the laboratory as well as on the pilot plant scale.

13.1
Power Alcohol

Due to the fact that Austria holds traditional expertise in the large-scale production of ethanol, efforts were directed mainly to evaluate the utilization of various raw materials with respect to their main importance in establishing the economic as well as the energy balances of the production processes (Vienna University of Technology, Linz University, Austrian Agroindustries). One of the aims was to work out schedules of continuously processing crops according to their availability throughout the year ('multicrop system'). As in most other countries, the strong dependence of costs on the availability of cheap carbohydrate raw materials precluded the implementation of extensive production programs such as that e. g. in the US ('Gasohol') or in Brazil. Thus activities remained in the range of pilot plant operation.

13.2
Biogas

With similar thoughts in mind, ambitious programs for biogas production from various waste materials were initiated (Graz University of Technology, Vienna

University of Agriculture) and various types of reactors were erected in several parts of the country [30]. Mainly on smaller agricultural sites, similar to the programs developed by UNIDO for developing countries, but with special industrial equipment, they were calculated to pay off within rather short periods of time.

13.3
Acetone-Butanol-Ethanol Fermentation

This process was economically feasible only under extraordinary conditions, as was the case during and after World War I. The history and decline of this process due to a variety of almost insurmountable problems are well documented (cf. [31]). Again, the oil crisis of 1973 initiated efforts to produce these products as alternative sources of chemicals and fuels. Among these, an interesting approach has been reported from a group from the Vienna University of Technology in cooperation with Massey University, New Zealand, comprising the use of immobilized cells and solvent removal by pervaporation [32]. Further efforts have been directed towards better insights into the physiology of the process.

13.4
Hydrolysis of Cellulosic and Lignocellulosic Materials

Following the well-known activities regarding acid hydrolysis in Germany in the 1930s, and the post-war investigations on enzymatic hydrolysis of cellulose in the US, a wealth of information accumulated through investigations in many parts of the world. Many of the investigations were devoted to methods of pretreatment of cellulosics and lignocellulosics. Austrian groups (Graz University; Graz and Vienna University of Technology; VOEST = United Austrian Steel Works) were also involved in respective studies [33]. The latter company erected a large pilot plant that was active until about 1994. As the main result, it may be mentioned that these activities also initiated renewed interest in scientific investigations, e. g. in details of the enzymology of cellulolytic reactions [34].

14
Environmental Biotechnology

Austria as a tourism country has high environmental standards. Environmental protection, therefore, is given appreciable priority there. Accordingly, research in the area of environmental protection and remediation is well accepted and supported. Only a few of the various activities will be reported here.

Industry, in particular the metal-processing industries, discharge heavy metals into the environment in different ways (industrial waste waters and sludges, waste gases, slags, fly ashes, filter dusts, etc.) and in astonishingly large quantities [35]. For years, the activity of a group at the University of Innsbruck has been directed at investigating and developing methods of biohydrometallurgy (biosorption, bioleaching) for such applications [36]. The primary objec-

tive in this endeavor was and is to decrease the content of metals (heavy metals) of such waste products, reducing the environmental hazards of disposal and at the same time achieving an increase in recycling efficiency. Research activities comprise screening of metal accumulating microorganisms as well as the development of special leaching processes that are adapted to the specific materials and the metals contained therein.

Another group of compounds that can become enriched in soil or water comprises persistent organic chemicals, such as pesticides or other substances derived from chemical processes. Among these, halogenated ring systems are important pollutants. A research group at the Vienna University of Technology [37] has successfully screened several bacterial strains that are able to decompose chlorinated phenoxyalkanoates and anilines as well as phenolic compounds.

At Graz University of Technology, soil remediation, especially with respect to pollution by diesel and higher hydrocarbons, has been studied [38]. The same group is also investigating the reduction of carbon monoxide in the exhaust air of road tunnels [39]. A biofilter system employing a mixed population of carboxidotrophic bacteria is claimed to enable a CO reduction of 10 g/m^3/h.

15
Pulp and Paper Biotechnology

Austria has a highly developed pulp and paper industry, thus several academic and research institutions have built up efficient research units. Pulping technology has undergone significant changes during the last decade(s). One of these was the switch from chlorine bleaching to e.g. oxygen bleaching, another might become the increasing application of biological (enzymatic) operations in pulping itself – hence biopulping. At the Vienna University of Technology, a research group started investigations into the action of white rot fungi on lignocellulosic materials. Besides these basic studies, attempts were made to develop a process of clarifying waste waters from chlorine bleaching. Dark colored chlorinated lignin decomposition products were successfully decomposed in a system containing immobilized cells of *Phanerochaete chrysosporium* [40]. Further investigations revealed that manganese peroxidase is the active principle in this system. Unfortunately, when chlorine bleaching was abandoned, the process became obsolete. Activities were therefore directed to the process of biopulping, i.e. the application of various basidiomycetes, e.g. from genera *Ceriporiopsis*, *Phlebia*, *Dichomitus* or *Phanerochaete*, for pretreating wood chips in order to increase the degree of delignification in the successive cooking process (cf. [41–43]). Research within this group was coordinated in an EU project "Oxidative Enzymes for the Pulp and Paper Industry" up to 1998 [44]. Another project dealt with the harmful deposition of resinous, hydrophobic compounds (pitch) from parenchymous wood cells in the course of e.g. papermaking. These substances were identified as unsaturated fatty acid containing triglycerides which could be hydrolyzed by common lipases and subsequently saponified [45]. It has been found that biopulping can also reduce pitching by about one third.

Two groups at this university have studied the possibility of wood protection using strains of *Trichoderma harzianum* as natural antagonists of typical wood

destroying fungi [46, 47]. With regard to the mechanisms of these processes, they came to different conclusions.

16
Products of Fermentation Processes

It goes without saying that fermentation research has also been continued over the last decades. One group at the Vienna University of Technology was among the first to introduce small personal computers as cheap and nonetheless versatile tools in fermentation analysis and control [48, 49]. Another group at this university has recently described a reactor configuration containing an expanded bed of synthetic pumice stone as a carrier for solvent producing clostridia in continuous operation [50]. At the Vienna University of Agriculture, several developments in the field of bioreactors (fluidized bed) for special tasks in cell culture have been achieved [51]. A novel type of microcarrier, combining hydrophobicity with a slight negative charge, has been developed which is especially suitable for fluidized bed operations [52]. These developments culminated in the establishment of a highly sophisticated computer controlled pilot plant [53].

16.1
Penicillin

The accomplishments of the 1950s were a strong stimulus to extend research in this area. Early activities (Biochemie Kundl, Vienna University of Technology) comprised investigations into the enzymology of penicillin biosynthesis [54] and its deacylation to 6-aminopenicillanic acid. These were followed by studies on the regulation and compartmentation of the individual steps of the biosynthetic process. They demonstrated that the first stage of penicillin biosynthesis occurs compartmented in the fungal vacuole, the relevant enzyme, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase being associated with the vacuolar membrane [55]. Successive genetic engineering experiments (Graz University of Technology, together with Biochemie Kundl GmbH) described the molecular characterization of the isopenicillin N synthetase gene [56] and revealed that gene expression of penicillin biosynthesis is regulated by nitrogen and glucose catabolite repression [57].

16.2
Organic Acids

As mentioned above, the large capacity of Austria's citric acid producing industry initiated research which was mainly performed at the Vienna University of Technology (cf. [11, 12]). Studies on the kinetics of this fermentation on the pilot plant scale led to a redefinition of this fermentation type (type II). Extensive investigations of the regulation and compartmentation of citric acid biosynthesis have shed new light on this process [58]. Improvements in downstream processing have also been reported from the above-mentioned group [59], as well as from a group at the Vienna University of Agriculture [60].

16.3
Polyhydroxyalkanoic Acids

The production of poly-β-hydroxybutyric acid (PHB) has been investigated right from the days of its invention by a group at the Graz University of Technology. Beginning with the aim of producing biodegradable substitutes to common plastics, processes have since been developed to produce tailor-made materials (e.g. copolymers) with versatile application properties [61].

17
A Step into Nano(bio)technology

The observation of regular layers at the surface of various bacteria gave rise to extensive investigations by a group at the Vienna University of Agriculture [62]. Apart from basic studies on the phenomenon of crystallinity and similar properties of these so-called S-layers and their ability to self-assemble into large-scale, coherent, two-dimensional arrays, main interest has focused on the practical applications of these structures. Initially, mainly applications as ultrafiltration membranes were envisaged [63]. Several possible applications have been investigated over the years and a wealth of information collected in a great number of publications and patents (e.g. [64, 65]). S-layers could be used as "immobilized matrices for biologically functional molecules or templates in the formation of ordered arrays of nanoparticles, which are required for nanoelectronics and nonlinear optics" or as "stabilizing and supporting structures for lipid membranes" or they could be "patterned by deep ultraviolet radiation and, after reinforcement, used as high-resolution resistors in silicon technology" [66].

18
Biocatalysis

It has already been mentioned when dealing with research on renewable resources that the study of polyglycane hydrolyzing enzymes has met special interest. An astonishing number of workers have investigated the general group of hemicellulases since it became apparent that these enzymes are important in the pulp and paper and in the food industry. Thus a wealth of information on xylanases became available. As an especially potent producer organism, *Thermomyces lanuginosus* was identified by a team at Graz University and University of Technology [67, 68]. Methods of large-scale production and details of applications have been studied in the respective laboratories [69.] A group at the Vienna University of Technology has purified and characterized the entire set of hemicellulases from *Trichoderma reesei* [70] and has also worked out means of optimum production. The two xylanase genes were cloned and the respective recombinant strains investigated for their enzyme-producing capacity.

The application of enzyme systems, combining coenzyme regenerating reactions, has been studied by a group at the Vienna University of Agriculture. Typical examples are the simultaneous production of gluconic acid and xylitol [71] or sorbitol [72], respectively, in membrane reactors.

In 1993, a "Special Research Program" (Spezialforschungsbereich) for Biocatalysis was established by the Austrian Science Foundation and the Ministry of Science and Research uniting the respective research capacities of the two Graz universities and combining them with other European groups. Main activities have been, and still are, directed towards areas such as:

- The production and modification of esterases by genetic engineering [73, 74];
- Characteristics of microbial lipases [75];
- Enzymatic and microbial hydrolysis of epoxides [76]; and
- Selectivity of microbial hydroxylation of organic compounds.

Cunninghamella blakesleeana has been found to be an efficient biocatalyst for stereospecific biohydroxylations of e.g. cycloalkyl carboxylic acids [77] and (also with *Mortierella alpina*) the respective cycloalkyl benzoxazoles [78]. The role of cytochrome P450 in these reactions is currently being investigated.

Within this program, an interesting approach to the synthesis of optically pure chiral cyanohydrins, which are important precursors for the production of valuable pharmaceutical compounds, has been elaborated: Hydroxynitrile lyases catalyze the formation of HCN and the respective aldehydes or ketones from α-hydroxynitriles. Certain plants can use these enzymes as a defense against tissue damage by possible predators (cyanogenesis). The reverse reaction is also catalyzed sufficiently by these enzymes yielding a variety of cyanohydrins [79]. The Graz group has isolated an (*S*)-hydroxynitrile lyase from *Hevea brasiliensis* and studied its specific synthetic potential [80, 81]. In two-phase systems, enantiopure (*S*)-cyanohydrins have been obtained in high yields and 98–99% enantiomeric excess [82]. The crystal structure of the enzyme has been elucidated [83] and the enzyme has been expressed in *Escherichia coli* as well as in *Saccharomyces cerevisiae* [84].

Within the same research program at Graz University of Technology, biocatalytic as well as product recovery processes applying supercritical carbon dioxide are being developed.

Several research activities are connected with applications in the food industries; e.g. at Graz University of Technology, a modified polyfructane splitting enzyme has been developed to improve the use of inulin as a food and fermentation raw material [85]. A group at the Vienna University of Technology, together with Bratislava University of Technology, has investigated optimum production of proteolytic enzymes of *Brevibacterium linens* involved in the flavor producing processes during ripening of the so-called red smear cheese [86]. A group at the Vienna University of Agriculture has investigated methods of production of various carbohydrate-converting enzymes, e.g. phosphorylases or a novel pyranose oxidase from *Trametes* [87].

19
New Medical and Plant Biotechnology

The new biotechnologies encounter low acceptance by both the public and governmental authorities, which is intensified by a rather tendentious coverage by the media and by various pressure groups. Thus, only medical biotechnology

is in a position to develop more extensive research activities. A recent inquiry referring research in medical biotechnology in this country lists more than fifty institutions from academia and industry with an impressive amount of projects ranging to such items as tumor vaccines and gene therapy [88].

A large group at the Vienna University of Agriculture has specialized in a number of aspects of mammalian cell culture. Novel methods of cell hybridization have been developed [89]. As mentioned earlier, a highly instrumented pilot plant has been established, mainly through financing from outside the university or government [90]. Advanced downstream equipment is provided as well. One of the main activities involves the production of monoclonal antibodies, especially of human antibodies against HIV-1 [91, 92]. These were obtained by cloning the information for human antibodies against gp41 (or gp120), the envelope proteins of HIV-1, into newly developed stable cell lines enabling the permanent production of these antibodies [90]. Antibodies thus obtained possess high neutralizing power against the respective epitopes [93 – 95]. The amino acid sequence of one of these epitopes has been analyzed (Glu-Leu-Asp-Lys-Trp-Ala) [93] and shown to be highly conserved in all HIV-1 strains isolated. This has also demonstrated the suitability of the respective antibody for diagnostic purposes. In the course of this work, the antibody/antigen complex of gp-41 of this virus has been crystallized and interesting images are available through the Internet [96a, b]. Successively, a chimeric influenza virus has been constructed that expresses the highly conserved amino acid sequence of gp41 [97]. Intranasal immunizations in mice with this virus induced a humoral immune response in respiratory, intestinal and vaginal secretions detectable for more than one year after immunization. This would provide a way for inducing long-lasting protective mucosal immunity [98]. Recently, an ultrasensitive HIV-1 antigen assay has been developed [99]. By using a novel chemiluminescent detection system, this assay can detect antigen positive samples 50 – 80 days earlier than seroconversion and equally earlier than the common antigen assays available.

Another group at this university has concentrated on the cultivation of insect cells with the aim of producing specific glycoproteins that e.g. display unique properties [100a, b]. Again, research activities are integrated in an information network (glycoscience network [100c]) accessible via the Internet.

Research in the new plant biotechnologies is impeded by the fact that in Austria release of important gene-manipulated plants into the environment has not been permitted so far, thus respective research is restricted to less eye-catching areas. At this point, mention should perhaps be made of the Austrian biologist Erich Tschermak-Seysenegg, Professor at the Vienna Agricultural University at the turn of the 20^{th} century, who together with Correns and de Vries in Germany rediscovered Mendel's genetic rules. By the way, Mendel himself was from Austro-Silesia.

Nonetheless, some activities may be mentioned, ranging from phytosanitary improvement to genetic manipulations mainly of fruit trees with the aim of inducing pathogen (virus) resistance [101, 102]. Examples are viruses of stone and pome fruit trees (e.g. plum pox virus [103]) and of vine. The complete nucleotide sequence of plum pox virus has been determined [104]. A second aim is to revitalize old strains of fruit trees and other cultured plants.

20
Other Genetic Engineering Applications

Although impeded by various constraints, several Austrian groups have exploited a number of areas for biotechnological applications. Work on antibiotic synthesis and immune biotechnology has already been described. Several of the main activities are related to gene expression in filamentous fungi [105]. Workers at Biochemie Kundl GmbH have cloned and characterized cyclosporin synthetase from *Tolypocladium niveum* in an attempt to create modified cyclosporins [106]. Two groups at the Graz and Vienna University of Technology have been, and still are, actively engaged in exploiting the potential of filamentous fungi as systems for homologous as well as heterologous gene expressions [105, 107]. An increasing part of the work on *Trichoderma* is devoted to this aim.

Austria, although better known as the country of music and magnificent landscapes, has made valuable contributions to science and technology in the past as well as in the present . Twelve Nobel Prize winners in the natural sciences, and discoveries such as e.g. the ship's screw (Ressel; 1827), the Welsbach burner and the cerium-iron flint (Auer von Welsbach; 1891 and 1907), the autopilot (Boykow; 1930s), or the LD process of steel production (Trenkler, 1940s) and most elegant automobiles (Porsche) – just to name a few – should be mentioned for demonstration. One of the aims of the present article was to show that, in the field of biotechnology also, several more or less significant achievements might be added to the record of human welfare and progress.

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Biotechnology in Hungary

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1 Introduction

Ever since ancient times, Hungarians have been interested in problems related to life. Our early interest in biology springs from our close relationship with nature: observation has become an essential life element and a generally adopted attitude in the field of science.

As expressed as early as one and a half century ago by our renowned natural scientist, Otto Herman (1863-1922): "Once the essential elements of a specific phenomenon are subjected to precise controlled observation, and preconditions governing repetition are defined, we will be in possession of an incredible tool; naturally, this is not the ultimate goal. But the more tools we have at our disposal, the closer they will bring us to our objectives, broadening the mind and leading to further progress. In all fields, we are trying to find the ultimate component, and once this has been revealed, the results may be realized."

A worldwide accepted view on biotechnology is that the phenomenon has always been known, but not recognized or given a name for centuries.

2 Preliminary Events in Biotechnology

In the course of history, man has gradually gained a closer insight into the world of natural laws and continually striven to gain control over its environment in order to utilize it for his own purposes. In the course of investigations we came into possession of information, which led – through the improvement of eating habits – to better living conditions.

Throughout hundreds of generations, methods involving biological activities had been applied as ancient trades within the family circle. In the course of time a group of "experts" emerged, baking bread, brewing beer, growing grapes, or making wine at a professional level. Finally, urbanization led to the foundation of various trade guilds.

During this period of time, hardly any changes in technology occurred, and if they did they were treated as a family secret handed down from one generation to the other, termed today as "know-how." The flow of information promoting development was an unknown idea. (The Hungarian Patent Office was established only in the middle of the nineteenth century.)

The establishment of guilds involved recognition and protection in practicing the trade. From the mid-seventeenth century, however, the system of guilds worked in our country as an "inhibitory factor" in the development of trade. With the appearance of charlatans, the system of guilds gradually declined and was abolished in Hungary in 1872. Finally, with the development of industrial enterprises, large-scale production has been introduced.

After the passing of thousands of years, with the discovery of Leeuwenhoek's microscope, microorganisms could be seen, and only 200 years later, in 1865, Pasteur gave a scientific description of the fermentation process. At this point, another achievement must also be mentioned: at a session of the Hungarian Society of Natural Sciences (13 November, 1861), a Hungarian chemist, M. Preysz,

reported on a procedure developed for the preservation of wine by heat treatment. His method was published, however, only in 1865, after the appearance of Pasteur's famous publication, thus his discovery could not be given legal priority [1].

It is generally not known that the term "biotechnology" was first used by a Hungarian expert, K. Ereky, in his book published in 1919: "The Biotechnology of Meat-, Fat-, and Milk Production in the Agricultural Plant" (in German) [2]. The title page is shown in Fig. 1. He stated here: "biotechnology deals with production methods where products are prepared from raw materials by means of living organisms," and in his opinion, "living creatures should be considered as biotechnological machines."

Although the term "biotechnology" was adopted worldwide only in the 1970s, it had been a generally accepted term used by Hungarian experts several years before. One example for this may be Raoul H. France, professor at the Agricultural Academy in Magyaróvár, who was first to apply "biotechnology" in industry – on the basis of his two patents – "biotechnical laws observed in plant- and animal life" and founded a plant, the "Edaphon Humusdünger-Werke" in Salz-

Biotechnologie

der Fleisch-, Fett- und Milcherzeugung

im

landwirtschaftlichen Großbetriebe

für

naturwissenschaftlich gebildete Landwirte

verfaßt

von

Dipl. Ing. Karl Ereky

Direktor der Viehverwertungsgenossenschaft ungarischer Großgrundbesitzer, Budapest.

BERLIN.

VERLAGSBUCHHANDLUNG PAUL PAREY.

SW., Hedemannstr. 10 u. 11.

1919.

Fig. 1. Title page of "The Biotechnology of Meat-, Fat-, and Milk Production in the Agricultural Plant" published in 1919

burg (then in the Austro-Hungarian Empire), in order to introduce a novel method of natural fertilization (Naturdüngung). He also stated that "biotechniques should be applied; I clearly felt I was facing one of the greatest challenges of mankind. Biotechniques will transform our whole civilization" [3].

After the era of Pasteur, biotechnology facilitated elaboration and further development of various manufacturing procedures. It led to the development of the fermentation industry, which promoted both food and pharmaceutical production, allowed the elaboration of antibiotics in 1940, a milestone in the history of mankind, and, finally, facilitated the production of vaccines, enzymes, amino acids, etc.

Another all-important revelation opening up new vistas in the field of genetics was announced on 25 April, 1953: the discovery of the DNA molecule. The first successful gene-transfer was carried out in 1973 (in USA), opening a new era of molecular biology and genetic engineering.

3
Traditional Biotechnological Methods in Hungary

Our first attempts at large scale biotechnological production were related to the production of food and other consumer goods (alcohol, tobacco), with a background dating back over 250 years (Table 1) [4]. By the end of World War I, the technologies of biological industries became widespread.

The use of microbiology in the pharmaceutical industry in large-scale production of vaccines was realized in 1912. The preparation of medicinal products of plant- and animal origin from living organisms started before World War II. A well-known procedure patented by a Hungarian pharmacist, J. Kabay (1896–1936) under the title: "A method for opium-alkaloid production from green poppy plants" in 1925 was realized on an industrial scale in 1927.

Elaboration of the "fermentation" technology also falls in this period, development accelerated, however, only after World War II. Outstanding results have been attained in the research and application of several other biotransformation procedures as well (e.g., in the transformation of antibiotics). Vitamin B_{12} production on an industrial scale was first introduced to the world in Hungary. Improvement of yeast strains and their application in the alcohol-, yeast- and wine industries became general practice in the 1960s, together with operation with up-to-date microbial methods for dairy products. Based on results attained abroad, several modern bioprocess plants started operation in the 1970s.

We were the first in the world to introduce beer-brewing with enzymes of bacterial origin and to establish a large plant producing both iso-sugar and alcohol from corn by the application of enzymes.

4
Special Industrial Methods Applied in Hungary

We would like to present here two special Hungarian projects relating to our special agro-ecological conditions.

Table 1. Initial steps of industrial production by biological methods in Hungary

Year	Site	Branch of production
1720	Szentgyörgy	Raisin wine
1787	Pozsony	Vinegar
1790	Pest	Vegetable oil production
1790	Kismarton	Wine cellarage[a]
1797	Fiume	Tobacco, cigar, snuff
1804	Ujlak	Brandy[b]
1808	Ercsi	Sugar on sugar-beet base
1825	Pozsony	Sparkling wine[c]
1844	Budapest	Beer-brewing[d]
1845	Temesvár	State-controlled tobacco
1853	Budapest	Yeast
1862	Budapest	Salami[e]
1872	Rohonc	Canning factory
1882	Szombathely	Cooperative dairy plant
1887	Füzfö	Starch
1912	Budapest	Innocula and vaccines
1923	Budapest	Lactic acid
1924	Budapest	Acetone-butanol
1927	Tiszavasvári	Alkaloid (morphine) from poppy seed capsules
1941	Kisperkáta	Glycerine
1946	Budapest	Penicillin
1951	Debrecen	Neomycin, oxytetracycline, tobramycin
1953	Budapest	Vitamin B_{12}[f]
1965	Ács	Leaf protein concentrate (VEPEX)
1969	Sopron	Brewing beer with enzymes
1971	Szabadegyháza	Corn-based HFCS and alcohol

[a] 1857: first wine competition in Hungary.
[b] 1850: number of large-scale alcohol distilleries: 5671 (with over 17 hl daily production).
[c] 1898: 17 sparkling wine production plants.
[d] 1851: 773 breweries.
[e] 1890: 13 salami factories.
[f] 1950s: 20% of Vitamin B_{12} world production.

4.1 Production of Leaf Protein Concentrates (LPC)

Our knowledge of leaf protein can be traced back over 226 years (Rouelle, 1773) [5]. The method of processing-preserving leaf protein suitable for human consumption was first patented in 1927 by Ereky [6], and a quarter of a century later some widespread experiments were carried out in the field in England [5]. An entirely different, novel procedure, the VEPEX (Vegetable Protein Extract) method was elaborated and the first leaf protein plant in the world was set up in Hungary in 1972 [7] (Fig. 2).

The production technology is closely related to raw material resources and supply. In our first experiments, annual protein production was altogether 500–600 kg/ha. By the 1970s, however, the "yield" was four times higher. With continuous green matter supply of our 180–200-day vegetation

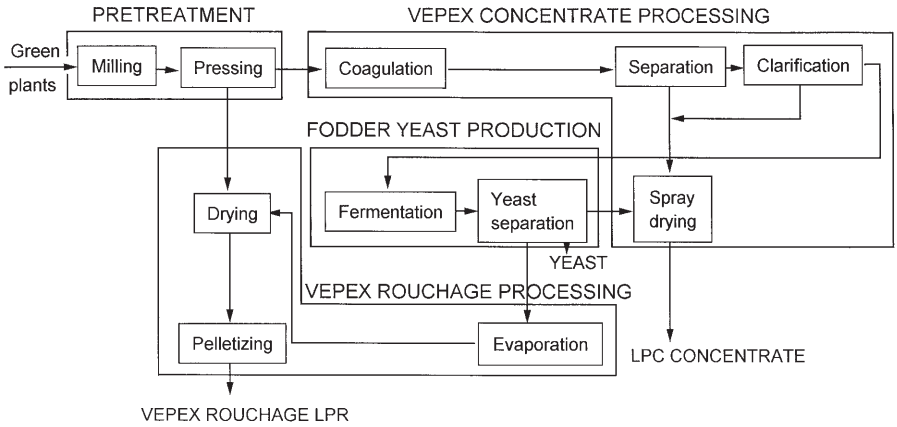


Fig. 2. Versatile Vepex LPC process

period, protein production amounted to 3000–4000 kg/ha, and in other temperature zones of the world (tropical zones) yields of 8000–10,000 kg/ha protein may be attained (10–15 times as high as protein yields obtained from fodder products) (Table 2).

Leaf protein obtained from a unit area gives the highest amounts of protein as well as most favorable results in essential amino acid yields, which would also make it profitable producing plants so-far not cultivated (e.g., *Atriplex*, *Tetragonia*, *Amaranthus*, etc.).

Table 2. Yield of fodder plants harvested as cereals or herbage crops per hectare based on Hungarian experimental results

Plants	Average yield		Dry matter		
	Ton/hectare	Ton/hectare	Raw protein Kg/hectare	Lysin Kg/hectare	Methionin Kg/hectare
Cereal fodder					
Corn	8.0	7.0	720	22.5	15.2
Soybeans	2.5	2.2	820	58.7	13.0
Herbage crops					
Alfalfa	49.9	12.7	2273	121.0	25.6
Autumn wheat	41.9	6.7	960	63.4	14.3
Field kale	40.1	4.3	720	60.8	7.3
Mixture of oats and vetches	30.0	6.3	886	45.3	10.0
For leaf-protein technology with continuous green plant supply (in approx. 200 days)	–	20.0	90.0	180.0	40.0

Characteristic products obtained by the procedure are [8]:

- chloroplast protein fraction (with amino acid content similar to that of soybean protein, applicable also as a substitute for extracted soy-bean meal or fish meal)
- cytoplasm protein fraction (applicable as a substitute for fish meal or milk protein, and, in the long run, also in human nutrition)
- fodder yeast
- syrup concentrate (used mainly as a supplement for fibrous residue)
- green meal or pellets (fibrous fraction applied as fodder meal)

Carotene- and xanthophil-coupled protein also play an important role in leaf protein production. Experts show growing interest in these coloring materials and then it is up to the customer to decide whether protein content or the coloring material is the decisive factor in evaluating the end-product.

4.2
Joint Production of Iso-Sugar (HFCS) and Alcohol from Corn

Hungary – as a typical corn-belt country with significant yields of corn production – is naturally interested in expanding and economizing large-scale industrial application of corn. Therefore, a technology for combined iso-sugar-alcohol production has been elaborated [9].

Figure 3 presents the production scheme of the Szabadegyháza Distillery, which has been in operation since the beginning of the 1980s, processing annually 150,000 tons of corn. In addition to starch, the following by-products are obtained: germ utilized in the vegetable oil industry, gluten for nutritional

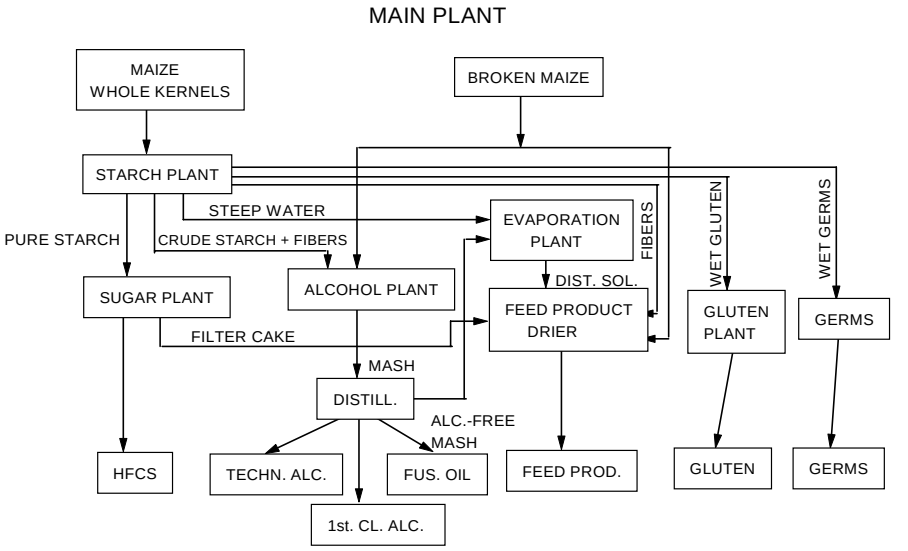


Fig. 3. General production scheme of the maize production complex in Szabadegyháza

purposes, and from starch: glucose and alcohol (depending on the demands), and from glucose: isosyrups (HFCS).

4.3
Brewing Beer with Enzymes

Brewing beer with enzymes is one of the practical applications of biotechnology realized worldwide on an industrial scale (Hungary was the first to introduce the technique industrially at the end of the 1960s [10].

In traditional brewing, malt can be replaced by unmalted cereals (barley, corn, rice, sorghum, millet, etc.) or other starchy substances (e.g., cassava, sago, yam- or arrow-roots) by simultaneous addition of appropriate amounts of protease and amylolytic enzymes with beta-glucanase effect.

In this procedure the technology and equipment of brewing only malt is applied.

Some benefits of the method are:

- the total cost of unmalted cereals and enzymes is much lower than for malt
- beer production can be increased without construction of a new malt plant
- countries dependent on malt imports may considerably reduce foreign exchange expenses by application of domestic resources and enzymes
- brewers' barley can be substituted by higher yield, less expensive raw materials

5
Evaluation of Biotechnological Research, Development, and Training in Hungary (1945 – 1980)

The training of specific areas of biotechnology (genetics, biology, microbiology, biochemistry) was carried out at the universities within the scope of the departments. Research was greatly dependent on the sphere of interest of the professor and was rather modest owing to the lack of funds and instruments.

After World War II, the trend of interest turned towards genetic sciences, an independent Institute of Genetics was established, but within a few years, B. Györffy's "school" of western orientation was completely wiped out by mandatory Soviet doctrines (Mitsurin). Those unwilling to adopt these views were excluded from academic life. International relations shrank to a minimum and it took several years for certain scientific areas and experts with "imperialistic views" to come to the forefront: these "unacceptable" ideas and results could be taught to university students only in terms of criticism.

In the field of plant biotechnology, tissue culture experiments are especially noteworthy, M. Maróti was a pioneer in education and introduction of these methods. It should also be stated here that, prior to the micropropagation of ornamental plants, experiments in growing orchids had been carried out as early as 1914 (M. Galambos), and large-scale cultivation started only half a century later (1968) [11]. In the 1970s, worldwide acknowledged novel results were attained in this field by the isolation of mutant cell lines and, based on this, in the reproduction of whole plants. Shortly afterwards, these methods of plant tissue

cultivation were also applied on behalf of agricultural producers for the isolation of virus-free carnations (pinks, Gerberas, grapes, potatoes, etc.).

The technique of genetic manipulation was first applied by the Szeged Center of Biology of the Hungarian Academy of Sciences (SZBK, MTA) (1974) and then carried out in different departments of the Universities (Department of Genetics, Microbiology, Biology, etc.). The method aroused interest in the industry as well, and in 1961 a National Network of Genetic Engineering was set up aimed at coordinating research in the field.

Pioneering experiments were carried out in the field of protoplast fusion (using bacterial and fungal protoplasts) leading to internationally recognized outstanding results. Significant advances were also made in the field of plant protoplast fusion and practical application has been initiated.

Investigations related to hybridome techniques and monoclonal antibodies started in 1978 and resulted in the preparation of hybridomes producing monoclonal antibodies in 1980.

The system of producing corn protoplasts was also elaborated in the SZBK and the first transgenic plant was produced by this research team in Hungary.

Transplantation of an alien gene into alfalfa was the first successful experiment in the world carried out with this important papilionaceous fodder plant. Experiments with nitrogen bonding were carried out, the isolation of Rhizobium genes was studied, and the molecular background of symbiosis has been analyzed.

After several years of experimentation, the Research Team for Cell Genetics of the Botanical Institute (SZBK) was first in the world to produce mutant plants resistant to herbicides, by methods of tissue cultivation.

Researchers in the Institute of Biophysics (SZBK) studying a hydrogenase enzyme isolated from a bacterium strain utilized in biogas production. With the aid of this strain, the efficiency of biogas production could be greatly increased (exceeding 5 – 10 times its original level) under laboratory conditions.

The Biological Station of the Institute for Immunology of the Eötvös Loránd University (ELTE) in Göd started an intensive study on cell-hybridization and cell-fusion in higher-order mammals in order to promote diagnostic investigations. As a result, antibodies prepared by the team were soon figuring in WHO lists.

Experts have been dealing with the problem of cattle- and sheep-embryo transplantation since 1976, when a program was launched for the annual import of several hundred heads of breeding-stock of high genetic capacity (mainly from the USA and Canada) in order to improve animal breeding in Hungary. For economic reasons the authorities in charge decided to build a plant suitable for local adaptation of the procedure, receiving import embryos and housing recipient or donor animals as well as a laboratory equipped with an appropriate surgical background. This was then the largest institution of this kind in Europe: 800 – 900 embryo transplantations were carried out annually and methods of embryo splitting have been introduced.

From the mid-1950s, in the training of chemical engineers the Department of Agricultural Chemical Technology of the Budapest Technical University (BME) introduced the training of unit operations of biological industries with practice

in pilot-plant experiments. Considering the interest shown, from 1971 BME and ELTE launched a jointly organized program offering a possibility for obtaining a degree in biological engineering.

By initiation of a national biotechnological R & D project, our primary aim was to promote the deepening of special training of engineers: under a post-graduate program we started the training of biotechnological engineers and offered R&D engineers working in industries one-year scholarships to research institutes in order to obtain further training in up-to-date research methods. We had more than 100 scholars working in these institutions, who joined in the work of investigations, and at the end of the term gave account of the results in a special essay.

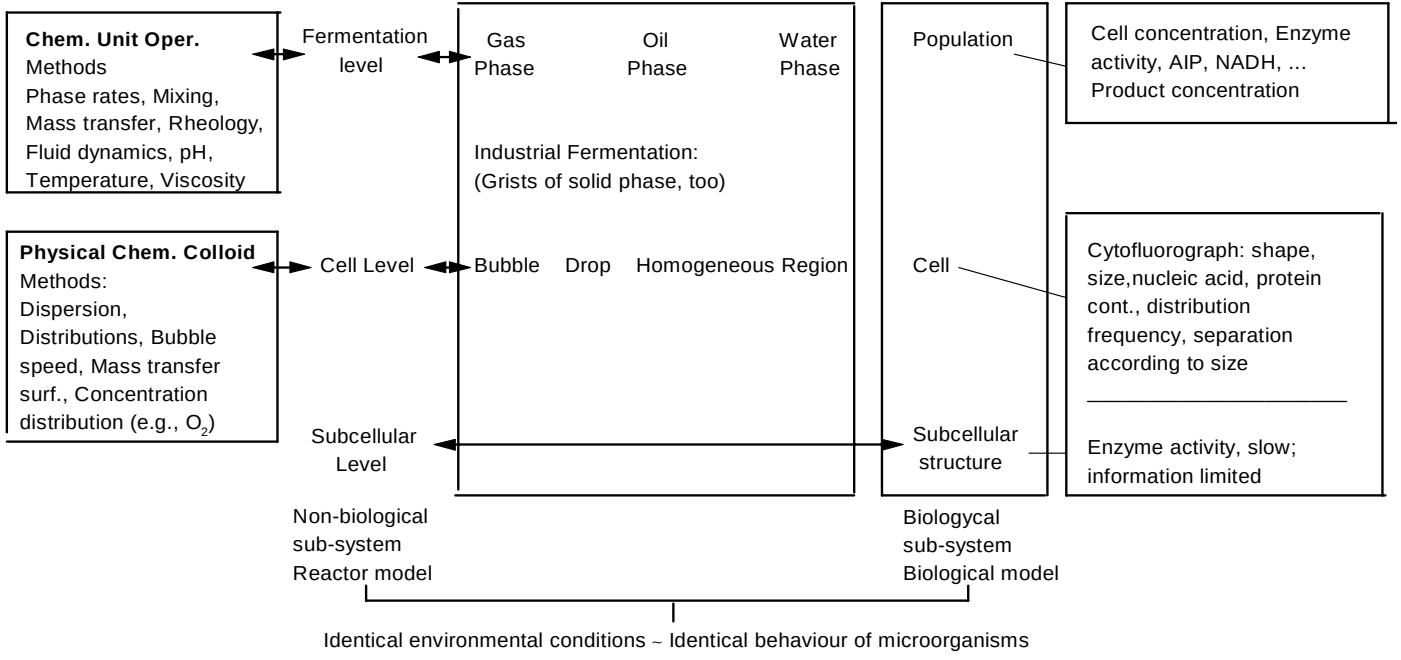
In the early 1980s, biotechnology training was introduced in the form of special courses in various university departments as well – Faculty of Natural Sciences, Agricultural University, University of Medicine, University of Veterinary Science, etc. In the course of time, interest in the subject has grown, but facilities were scanty: the import of modern instruments, machines, tools was strictly limited (with low and hardly available foreign exchange quotas), and prospects for establishing pilot-plants were bleak.

The demand for controlling and optimizing bioprocesses has been an issue for many years. In the early 1970s (when such equipment was not available) the Fermentation Group of the Department of Agricultural Chemical Technology BME was the first in the world to develop research bioprocess equipment with computer control, including computerized process control (Fig. 4) [9]. Another pilot-plant scale system with a similar function has been built in the Biogal Pharmaceutical Factory. This close link between biotechnology and computer techniques has then been put into practice in all plants dealing with fermentation processes in Hungary.

In this period, activities in this field were greatly promoted by scholarships to research institutions in the western world, offering researchers the opportunity to learn novel methods and participate in research investigations as well. As a result, their efforts gained international acknowledgment and personal relationships led to regular cooperation maintained for many years. Cooperative work between the individual institutes first started under inter-governmental agreements, with poor domestic background support (the ratio of 50:50 envisaged could be considered only as a nominal figure).

In spite of this, considerable participation in international scientific life has been attained: Hungarian experts began to participate in international congresses and soon were among the founders of the European Federation of Biotechnology.

Significant changes in this field were sparked by the Hungarian Academy of Sciences, where researchers and science-organizers in the 1960s had the opportunity to observe great international development in the field of biological sciences. Bruno F. Straub managed to persuade the government to establish a research institute in Szeged, dealing specifically with basic research. Activities in the SZBK started in 1970, with a staff of approximately 150 researchers working in 5 institutes (Genetics, Plant Physiology, Biochemistry, Enzymology, Biophysics).



Unified Mathematical Model (For process control)

Objective: analysis of the composition of the phases: developing on-line measurement technique fermentor-interface, instrument (computer)
Difficulty: great number + variety of components/selective analytical method not sensitive to interferences separation before detecting
sterile conditions (built-in sensors, airtight scaling, endurance of strerilization)
on subcellular level: biochemical informations are limited

Fig. 4. Instrumentation, automation of bioprocess

6
National Research-Development Program for Biotechnology (1984 – 1990)

In 1982, on the initiative of the Council of Ministers, a report was issued on biotechnological research, application of the results, and on conditions and prospects of development in biotechnology. According to the report, we were able to take part in international competition in biotechnology, and development in this field was also significant for the future development of our country. The report also contributed to the fact that biotechnology played an important role in converting the economic structure and increasing competition in Hungary. Up to the 1990s, biotechnology was noted in all documents on economic policy as being an important area of development, and in target projects and other departmental programs it was given special treatment and support (grants received from government sources (1983 – 1990) amounted to 4.5 billion Hungarian florints, i. e., US $ 50 million according to the current exchange rate).

According to a survey conducted in Hungary in 1982, R & D was being carried out in certain fields of biotechnology in 40 different institutes, with 400 researchers, development engineers, and physicians participating in the work. It has also been stated that "biotechnological research and production in Hungary has a considerable background. At the same time, however, the significance and standards of R & D activities were no longer sufficient for further efficient production development. Therefore, a selection of tasks, concentration and acceleration of activities in promising fields were required."

Therefore, governmental authorities engaged in biotechnology (MTA, State Office of Technical Development (OMFB) and experts of the Ministry of Agriculture, of Industry and of Health) suggested and adopted for the period 1984 – 1990 a national R & D project under the title: "Research and Development in the application of new biotechnological methods."

Considering the size, economic potentials, and current academic capacity of our country, conducting R & D activities in the entire field of biotechnology cannot be considered a realistic goal. With critical approach and appropriate selection, we have decided on the study and solution of medium-range tasks with applicable results and some long-range projects with promising prospects in international competition.

The 850 experts (from various research institutes, university departments, industrial plants, agricultural units) participating in the work considered as their objectives (Fig. 5):

- Acceleration of current activities supporting biotechnology in genetics, biology, microbiology, biochemistry, and techniques
- Production and product development based on recent results which may facilitate better satisfaction of domestic needs, utilization of foreign trade potential, and the improvement of economic efficiency

Subprograms:

- Research and development of biotechnological procedures based on protoplast fusion

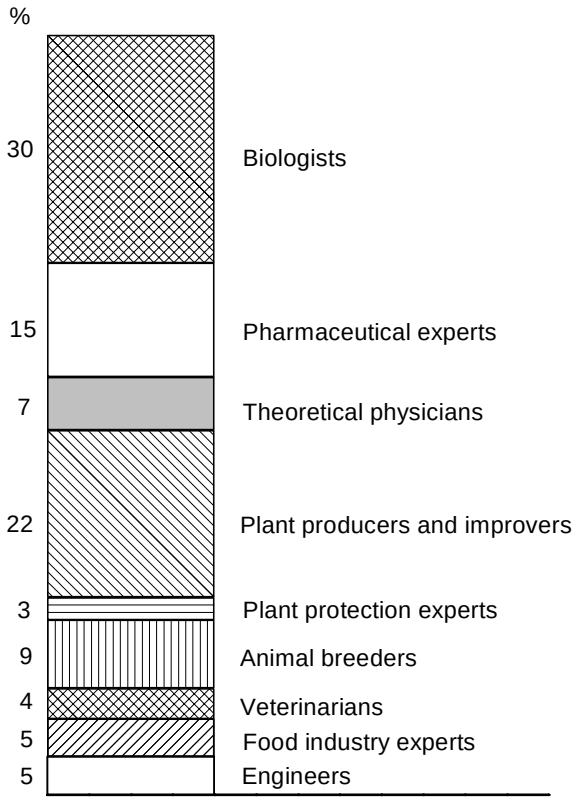


Fig. 5. Researchers engaged in biotechnological areas (850 persons)

- Elaboration and application of more efficient technologies facilitated by gene-recombination
- Bioengineering activities for the study of operation problems and for the application of novel techniques
- Further development of protein-biotechnologies, elaboration of new methods of quality improvement

Under the program, close cooperation has been established between university-research laboratories-industrial plants and experts in genetics, medicine, and economy; progress in these fields was regularly evaluated and the results attained were shared with other related areas, even in a "semi-finished" state.

6.1
Results of the National Research-Development Program

The data below have been collected from research reports and accounts of the Technical-Scientific Committee of the program, which have not been published [12].

6.1.1
Advances in Pharmaceutical Research

- Great efforts have been made to develop the preparation of antibiotics by novel biotechnological methods. In order to increase productivity of the wide spectrum of *Streptomyces* strains, experts developed a novel vector construction in the *Cephalosporium* fungus. *Streptomyces* vectors were also applied for the transformation of *Micromonospora* strains. By optimization of the essential parameters of fermentation, 15 – 20 % higher *Cephalosporin* C production has been attained, compared to the results of the original technology.
- In order to improve the production of gentamycin applicable against penicillin resistant microorganisms and gram negatives, new cloning vectors were developed for the application of *Micromonospora* strains. Genes affecting biosynthesis were cloned in the producing strain in order to increase productivity. By further development of the mutation-selection of clones, productivity of the strain could be raised by 40 – 60 %.
- With a commercially obtained vancomycin-producing microorganism strain, which inhibits cell-wall synthesis, by appropriate biotechnological methods and a modified fermentation technology, production yields were 30 – 40 % times as high as the original figures attained.
- By application of self-fusion, natural impairment in the productivity of *Streptomyces fradiae*, which produces neomycin inhibiting the protein synthesis, could be prevented and recombinants with higher productivity than the parent strain could be isolated. By selecting appropriate strains, we were able to improve greatly the efficiency of industrial fermentation.
- Through the development of the strain *Str. tenebrarius*, under optimized conditions of the technology, tobramycin production of *Staphylococci*, an effective anti-*E. coli* agent, could be raised by 20 %.
- Significant research was conducted with a view to developing and introducing hirudine (an anti-coagulant) as a medicine in Hungary. In hardly more than 30 months, by application of the recombinant DNA technique, and with the use of *E coli*, *Saccharomyces* and *Streptomyces* host organisms, various plasmid constructions were prepared, and from yeast-ferment-broth an HV-1 hirudine component was obtained, which allowed industrial-scale production of hirudine.
- Successful technological adaptation and pilot-plant scale development was attained in the field of the immune-suppressive cyclosporin-A production.
- Further development of the optimization of metabolism-controlled fermentation served the purpose of more efficient production of erythromycin and aminoglycoside antibiotics (nebramycin, neomycin). In a new bioreaction system, simultaneous addition of 8 different substances and continuous measurement and control of 11 parameters were solved by on-line computer control.
- Development of an inactivated vaccine for swine fever was also followed with great interest. Virus strains well adaptable to cell culture have been developed. The inactivated vaccines proved to be just as effective as living-virus vaccines.

- Procedures for preparing new steroid intermediates and ergot alkaloids have been further developed. A new method of bioconversion was elaborated for the degradation of sterine side-chains, which allowed selective degradation of the side-chain without damaging the sterane skeleton.
- In the case of ergot alkaloids, productivity of the strains *Claviceps purpurea* could be improved by protoplast fusion, which resulted in more profitable production.
- For better diagnosis and treatment in medicine, three kits have been prepared:
  - For the determination of IgG and IgN-type antidotes against the Cytomegalovirus (marketed also as export products)
  - Fibronectine for the determination of protein
  - IgG antibodies for the isolation of Herpesvirus-1 and Herpesvirus-2
- Furthermore, approximately 100 well-defined new monoclonal antibodies have been prepared. More than one-third of the hybridoma cell line, producing about 150 monoclonal antibodies is stored in the National Hybridoma Cell-bank (Immunological and the Biotechnological Laboratory, University of Medicine, Pécs), which was set up specifically for these investigations. (Most of these cell lines can be utilized in human-, animal- and plant medicine.)

6.1.2
Results in Plant-Improvement and Production

- Outstanding results have been attained in the technological development of micropropagation on plant tissue cultures (Propamatic system), in the elaboration of necessary tools (production organization of the tools) and automation of the operational steps. The system allows annual production of millions of propagating agents from a single plant. (The system has been patented in 16 countries.)
- In the development of the first wheat species produced by biotechnological methods, patented as "GK KINCSO", genes resistant to mildew, powdery mildew, and stem rust were incorporated into wheat improvement raw materials.
- In the improvement of high-protein fodder wheat, higher grain production was attained, and protein content could be raised by 1.5% (approx. 13–14% relative ratio).
- An efficient protoplast plant system has been developed, based on which interspecific hybrids not available by traditional improvement have been produced by a method of somatic hybridization.
- A successful micropropagation technique has been elaborated for the production of asparagus, a rather delicate and valuable product.
- In vitro cultivation of "blue grape species," where color intensity can be ensured by cross-breeding, has been successfully realized, resulting in shorter cultivation time, virus- and bacterium-free plants resistant to several fungal diseases, and resistance to unfavorable environmental conditions.

6.1.3
Results Attained in Animal Husbandry

- In ruminants suffering from contagious diseases (leucosis, brucellosis, chlamidia, etc.), successful results were attained by embryo transplantation. The method can be further developed by embryo splitting, producing genetically identical twins.
- A worldwide acknowledged novel technology involves inactivation of antinutritive factors of soybean by microbiological methods. In the joint silage of a mixture of freshly harvested corn and raw soybean (CSM), under appropriate conditions, factors hindering the digestibility of nutrients of soybean can be inactivated in a bacterial process in a few weeks.
- For satisfying amino acid requirements of ruminants, experts developed a procedure, as a result of which the degradation of administered synthetic amino acids takes place in the small- and large intestines and not in the rumen. On the basis of microcapsule coating, the method of dimethionine coating was elaborated, and a plant with several tons of capacity has been established. Daily administration of only a few grams of "protected methionine" raised milk production by 1.2 – 1.5 l/day.
- In earlier years, in fresh-water fish production (e. g., carp), reproduction by parthenogenesis, producing exclusively female offspring, was a significant method. Recently, the production and storage of sex-specific carp sperm, as well as the conversion of male eggs by hormonal treatment has been solved.
- An entirely new incubation technique was developed for "water-free hatching of fish eggs". The new method involved: low water and energy consumption; survival at least in ratios attained by traditional incubators; sterilization without malachite green, antibiotics or other banned chemicals; minimal labor; and hatching at an optimal time. The incubator works for both fresh-water and sea-water fish eggs – preferably for most cultured species.
- Breeding of transgenic animals: the gene-map of cattle and rabbits has been charted, the hormone growth gene of cattle was isolated, the cDNA clone bank was set up, etc. Various methods were elaborated for producing transgenic animals, and offsprings originating from more than two parents, "chimeras," were successfully brought into being.
- Most recently, a system of microcarriers was introduced in order to promote vaccine production in animal hygiene. A virus for "Aujeszky's disease" was cultivated on two cell lines, on microcarriers with titer values 10 – 15 times as high as gained by traditional methods.

6.1.4
Results Attained in the Food Industry Field

- An enzyme product for cell wall degradation was developed, by which the number of living microbial cells in meat products could be reduced. The product has been tested on pilot-plant scale with good results.
- Successful attempts have been made for further development of brewer yeast strains, which increases the rate of fermentation, improves aroma formation

and the quality of beer, and incorporates new properties, flocculation capacity, killer-property, etc. in yeast. The killer-strains developed by the system reduce lagering time and increases the economic efficiency of production.
- In the food production field – and product development – starter cultures, which greatly increase the efficiency of fermentation, have been produced. The technology was improved mainly by application of lactic acid-producing bacteria and yeast fungi, which led to higher quality products.
- In champagne production, for the development of high efficiency yeast strain (with higher autolytic properties) well adapted to cold, the haploid line of *Saccharomyces cerevisiae* yeast strains was treated by mutagenic UV irradiation. Then, with a hybrid obtained by protoplast fusion, the rate of sugar decay in filling wine has been increased. As a result, the amount of dry matter yield obtained was three times as high.
- The development of waxy corn species offered better potentials for the production of starch derivatives in high demand in the world market. The technology for the production of waxy starch-acetate has been elaborated.
- A "gene bank of microorganisms" has been established in cooperation with the University of Horticulture and the Food Industry in order to make available to researchers and industrial experts a strain collection or gene bank of industrial microorganisms. Under the program the institution acquired, in the course of patenting, a legal status as an international depositary.
- Noteworthy results have been attained also with our strain collection of lactic acid bacteria, of lignocellulose-degrading microbes, and their technological application.
- In human nutrition, e.g., for the elimination of unfavorable immunological reactions, alimentary allergies (milk-protein-, food-, flour sensitivity), the original protein structure has been changed by enzymatic peptide modification. By application of the method, hypoallergenic products can be prepared from various food proteins (milk, eggs, soybeans) by which the nutrition of allergic patients, mainly infants and small children, can be greatly improved.

6.1.5
Results Attained in Environmental Protection

- Biological waste water treatment has been intensified by application of special microbe preparations with a capacity for higher degradability. A "polyfunctional population" compiled from different bacterium strains, a mixture containing a wide metabolic spectrum mainly of fat-degrading organisms, has been prepared. By application of 1–2% inoculum, the efficiency of waste water treatment could be raised from 40–60% to 95–97%.
- By a systematic study of selection mechanisms of immobilized mixed bacterial cultures, objective methods have been developed for the description and control of microbial adhesion to inert surfaces for denitrifying organisms and aggregation of methanogenic cultures.
- Applications:
  - A denitrifying culture of outstanding specific activity has been reached for use in fluidized bed reactors (more than 10 kg NO_3-N/m^3d). Full capacity

and removal efficiency could be maintained without substrate inhibition up to the extreme influent concentration of 900 mg NO^3-N/l.
- A new kinetic description of a granulation process and start-up strategy has been conceived for acetotrophic methanogens applied for anaerobic UASB reactors.
- On the basis of a new method correlating the biomass concentration with the vertical pressure gradient of the fluidized bed, a comprehensive optimization and modeling procedure has been elaborated for fluidized bed bioreactors.

6.1.6
Development of Research Institutions

R & D potentials between basic- and applied research in agriculture had arisen during the half term of the national project. Therefore, a government decision has been reached to establish a research center associated with the University of Agriculture in Gödöllö. Accordingly, the Research Center for Agricultural Biotechnology was established, started operation in 1988, and launched a program based on both basic and applied research in plant biology. Noteworthy results were attained in the regulation of genes controlling the development of potato tubers and in the elucidation of the molecular background of resistance to potato-rotting and potato beetles. For further development of plant transformation systems, new patented procedures and instruments have been elaborated. As a result, transgenic rice has been produced and cells of woody plants were successfully transformed. Researchers of the Institute initiated investigations in molecular plant virology and determined the primary structure of several plant viruses from the Hungarian flora (cauliflower mosaic virus, cucumber mosaic virus, plum pox virus, potato Y virus, tobacco necrosis virus, etc.). For protection against plant viruses, cross-protection induced by the capsuleprotein-virus was successfully attained in relation to several host plant viruses. A unique research project involved the study of parasitism in higher order plants by molecular biology, molecular taxonomy, determination of micotoxins with a trichotechene skeleton, our first results in molecular plant pathology, which also won international recognition [11].

In the meantime, another institute of the MTA, Agricultural Research Institute in Martonvásár, developed into an important center of biotechnology, carrying out activities both in plant improvement and basic research. Researchers achieved outstanding results mainly in the field of in vitro culture and manipulation of plant (wheat and corn) gametes, one of the most exciting areas of plant biotechnology (artificial insemination, haploidization, microspore- and embryo transformation, etc.). Under special conditions, diploid wheat and corn lines have been developed, a method also successfully applied in the selection of aluminum-tolerant genotypes. The institute also played a pioneering role in the application of molecular DNA markers in plant improvement.

Outstanding, internationally acknowledged achievements were attained in the Research Institute for Cereals, Szeged, in the elaboration of tissue-cultures facilitating the genetic transformation of corn, wheat and rapeseed. The corn-

protoplast transformation system developed and patented under a cooperative project by Hoechst (Germany) and MTA SZBK (Hungary) is also remarkable. The results achieved in the development of androgenetic systems of corn and wheat are also significant. Correctness of the approach has been verified by two novel wheat species (GK Délibáb and GK Szindbád). The use of molecular markers for the improvement of both species has been initiated, for the present, only on moderate scale.

The Veterinary University set up an experimental unit in Üllö with a view to gradually extending biotechnological methods.

6.2
Experience in Program Coordination

From the viewpoint of science history and research organization, establishing personal relationships for continual exchange of views between R & D experts has been considered a task of vital importance. In addition to individual "gray matter" and academic capacity, this served as a basis for the achievements attained and, in some cases, even compensated for the lack of funds.

Our experience in the field may be summarized as follows:

- An essential point was to establish the practice of "joint consultation" between experts (agriculturists, chemists, mechanical engineers, biologists, physicians, microbiologists, economists, etc.) working in different areas under the control of various ministries. Therefore, three-day consultations (brain-storming) sessions were organized offering open discussions of the different topics, methods, ideas and potentials. In the course of these 72-h sessions, initial segregation between special areas, possible conflicts and jealousy were removed, to be replaced by mutual understanding and confidence in the joint project.
- The order of application was fully observed, anyone could submit a proposition, and the method of invitation for certain tasks has been adopted. Competing parties joined in the solution of tasks and cooperation has been established.
- Naturally, such positive results could not be attained in all areas of the project, but failure was also considered an important point to discuss, as it put an end to further unsuccessful attempts. In critical issues, with knowledge of the negative results, failure could often be reversed (even in the case of sensitive attitudes towards creation – evaluation – organization).
- A fundamental principle of the project was that successful research requires mutual confidence between the researcher, coordinator (research organizer), and the institution sponsoring the program (in our case OMFB and/or the government).

7
Biotechnology and the Society

The government-sponsored a biotechnological research-development program finished in 1990. Controlling the recovery of expenditures on research-development is still not the general practice in Hungary: a survey of the biotechnolo-

gical R & D program has been completed. Comparison of the expenditure and results showed that the program was highly successful: direct economic yields attained by application of the R & D project exceeded the expenditures already at the conclusion of the program. Other indirect results of biotechnological development, including "social benefits" gained in the field of diagnostics, therapeutics, and environmental protection, as well as benefits arising from the development of the infrastructure, education, and professional training, although hard to express numerically, may be considered as significant achievements.

It has been verified that biotechnological activities are indispensable requirements for improving the "quality of life," and that human problems of diagnostics and therapeutics should receive due consideration. In this respect, direct economic evaluation should be placed in the background: prevention, rapid and safe diagnostics and treatment, etc., offer much higher benefits than can be gained materially.

Regarding the considerable (realistic or exaggerated) social aversion experienced in various European countries, including Hungary, to food raw materials and products produced by biotechnological methods, and/or objection to the dangers involved, upsetting the balance of nature, the spread of information and dissemination of knowledge on the topic as well as legal regulation in the field are of vital significance.

After many years, as a result of serious efforts, "activities in genetic technology" have been regulated in an Act brought by the Hungarian parliament in 1998 (Act XXVIII, 1998) [13] and, accordingly, the "Committee for Evaluating Genetic Technological Procedures" has been set up.

In terms of the law, approval of the Committee is required for:

- utilizing, issuing, marketing, importing, or exporting organisms modified by genetic engineering (except for research purposes)
- modifying natural organisms by genetic technology (except for experimenting)
- establishing new laboratories performing modification by genetic technology

According to the law, if permission is granted, "the user must employ a person in charge of observation of the law and regulations of the Act, as well as supervision over the project in order to prevent activities harmful to the health and detrimental to the environment."

Based on these regulations, public control of biotechnological activities has been realized.

In principle, the problems of patenting biotechnological procedures have also been solved; however, the issue of patenting cell lines is still an open question. The basic problem is that in Hungary (as opposed to several other European countries), there is no potential for patenting cell lines produced by recombination techniques or by cell hybridization.

8
The Position of Biotechnology at the Turn of the Millennium

After the collapse of the communist political-economic system in Hungary in 1990, an entirely new situation has arisen: privatization of governmental enterprises, recovery of agricultural land as private property, extension of foreign enterprises and investments, etc.

Naturally, the change over to a market economy had an impact on scientific life as well: the staff number of researchers has decreased by 40 – 50 % and, owing to changes in ownership, several industrial institutions and laboratories have closed down. Funds available for research and development have greatly decreased; while amounting in the 1980s to 2.1 – 2.3 % of the GDP, the amount subsequently dropped to 0.5 – 0.8 % [14]. This meant that, under the new system of competition, chances for financial support in biotechnological research are on the decline; R & D resources in the field of biotechnology have decreased (government support as well as company expenditures on biotechnological research and development greatly reduced). For at least half of the research staff in Hungary, sufficient funds for justified scientific or economic activities are no longer available, either as government support or calls for charity.

Unfortunately, we had no "safety net" to protect us, and developments in biotechnological R&D suddenly broke down, partly due to the lack of material resources, and partly to the constantly spreading new attitude of "solving only the most urgent daily problems." Therefore, the question may justly arise: How to go on with biotechnological R & D and production in Hungary? The answer to the question requires due consideration and evaluation of the following issues:

- Natural resources
- Economic situation
- Academic capacity
- Potential role in international life.

A short review of these questions follows.

Natural resources – the total area of the country covers 9.3 million hectares, 72 % of which is arable land currently under cultivation. According to a survey in 1980, annual biomass production amounts to 53.4 million tons (in terms of energy content 941 PJ). (1 PJ = 10^{15} Joule); approximately 53 % of this is grown as the main product (with 28 % used as food products and 72 % as animal feed) [15]. On the whole, the total area of arable land, gardens, orchards, vineyards, is 0.7 hectares per capita, considerably exceeding the average values of other European countries. According to another survey, agro-ecological potentials of the country are very favorable [16]: long vegetation time, high thermal values, long periods of sunshine, configuration of terrain and soil, possibilities of natural endowments for special regional production, etc. Agricultural production exceeds domestic needs by about 40 – 50 %.

Economic situation – economic recession experienced in the first decade after the change of the political system in our country may be attributed to both internal and external effects, but this has been successfully overcome. Govern-

mental previews envisage an annual economic growth of 5–7%, considerably higher than average European figures. This is, however, an essential requirement for meeting the standards of the European Union.

Academic capacity – the qualifications of our research staff are satisfactory. With the number of university students doubling in recent years, we also have promising prospects regarding age distribution. Foreign interest in qualified experts is considerable and Hungarian experts invited to work in research institutions or industrial companies abroad have attained successful results and acknowledgment.

Potential role in international life – after 40 years of isolation, with the collapse of the "iron-curtain" there are no more obstacles to our establishing relationships, primarily with European countries. These international relationships are further extended and strengthened by increasing activities in economic policy: the volume of trade shows growing trends both in extension and material values. The success of the 1997 EFB Congress in Budapest also points to efficiency in international cooperation. Evaluation of the situation shows that conducting biotechnological activities is of vital importance in Hungary. In addition to legal and safety regulations, the dissemination of knowledge, and proper information on biotechnology must be an everyday project. Science and research have actually never worked against social interests (even strategic military development is not always offensive, it may also serve defensive purposes). Therefore, biotechnology must be given wide public approval in the future. The driving-force of science can manifest itself, however, only if it enjoys public confidence and economic support, inspiring further successful research and higher creativity.

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Biotechnology in Switzerland and a Glance at Germany

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The roots of biotechnology go back to classic fermentation processes, which starting from spontaneous reactions were developed by simple means. The discovery of antibiotics made contamination-free bioprocess engineering indispensable, which led to a further step in technology development. On-line analytics and the use of computers were the basis of automation and the increase in quality. On both sides of the Atlantic, molecular biology emerged at the same time, which gave genetic engineering in medicine, agriculture, industry and environment new opportunities. The story of this new advanced technology in Switzerland, with a quick glance at Germany, is followed back to the post-war years. The growth of research and teaching and the foundation of the European Federation of Biotechnology (EFB) are dealt with. The promising phase of the 1960s and 1970s soon had to give way to a restrictive policy of insecurity and anxiousness, which, today, manifests itself in the rather insignificant contributions of many European countries to the new sciences of genomics, proteomics and bioinformatics, as well as in the resistance to the use of transgenic agricultural crops and their products in foods.

Keywords. Antibiotics, Contamination-free mass culture, Molecular biology, Genetic engineering, Computer application, On-line analytics, Process automation, Transgenic plants, Food from genetically modified crops, Restrictive policy, Ethical concerns

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1
From Fermentation to Modern Biotechnology

Biological systems in the flora and fauna, as well as microbes to transform substances, have been used by man since the time of the early cultures. In the course of centuries, the preparation of bread, beer and wine reached a remarkable standard in the advanced civilisations of Asia and Egypt. Many present-day historical overviews label this early phase of technical development biotechnology, though it was based on spontaneous reactions [1]. The typical examples are fermentation with alcohol or acidification (milk, vinegar, butyric acid, yoghurt); the latter process is also called "Gärung" in German. It is quite common practice and includes metabolism and technical processes. Modern biotechnology in contrast is based on gene technology, massive data processing and highly sophisticated analytical processes. It has become calculable and reproducible, making – apart from microbes – use of enzymes, cells or groups of cells of human, animal or vegetable origin as catalysts, quite apart from microbes. For a process in medicine, agriculture, industry and the environment to be classified as biotechnology, it must involve genetically engineered cells, tissue or plants, and/or high-tech engineering. Biotechnology today goes beyond the old spontaneous processes and has little in common with the former incomplete oxidations.

The first steps towards a rational use of microbes were made possible by the work of Pasteur, who in the 19th century refuted the idea of spontaneous generation and thus made the introduction of pure cultures and pasteurisation possible. Progress was made in medicine (vaccination), industrial enterprises (application of yeast and bacteria) and fermented food and beverages by application of microbiology. The fundamental role of microbes in the metabolism was slowly recognised, and people were impressed by the elegance of biological synthesis and the methods of biodegradation. The rational approaches of that time contributed towards a general understanding of biochemical metabolism in microbes, man, animals and plants.

The progress made by Pasteur's microbiology reached Switzerland very early. In 1892, the first course of lectures in dairy bacteriology was introduced at the Department of Agriculture of the Swiss Federal Institute of Technology (ETH) in Zurich. This course of lectures as a minor subject was given by F. von Tavel.

This was the beginning of a remarkable development of microbiology at ETH in Zurich. In 1906, the Institute of Agricultural Bacteriology, and in 1944, the Institute for Dairy Technology were created. During the war, a shortage of master brewers was felt, and this initiated the introduction in 1948 of Fermentation Biology at ETHZ, which in the sixties developed into Technical Microbiology.

The research activities of this Institute for Agricultural Bacteriology and Fermentation Biology were geared to the needs of the economy in those cellulose, times of hardship. Ethanol and feeding yeast processes on the basis of wood sugar (xylose) and metabolic studies of the acetone-butanol formation, but biological degradation of wood were also of prime interest. Process technology in the proper sense was not pursued, although wood hydrolysis was technically fairly limited, even after two wars, and although, in peace time, biological processes

were subject to keen competition from chemical syntheses. Simple molecules such as ethanol or solvents were soon produced without the help of microbes. There remained the classical fermentation processes in the preparation of foodstuffs such as baker's yeast, cheese, wine, beer, vinegar, and citric acid. But progress in natural product chemistry opened up new vistas in pharmaceutical products to the chemical industry.

Tubs and vats reflected the state of art before World War I. Agitation vessels with active aeration were used in the production of yeast (M. Röhr [2]). In World War II, mixing and stirring posed serious problems in mass production of ethanol. The German plants for the production of ethanol in Tornesch, Holzminden and Dessau never got beyond 70% of the planned output. One ton of wood yielded 160 kg of ethanol only. In peace time and after careful scrutiny of their economic viability, these plants were closed. In Switzerland, the production of ethanol from wood could not cover the investment and running costs. Ten years after the war, the Swiss voters decided to withdraw government subsidies from the plant in Ems, which led to its closure.

Only the introduction of processes to produce antibiotics led to an important leap in process engineering. In 1940, Chain and Florey, in Oxford, noted the antibiotic effects of penicillin in vertebrates for the first time. The production in stirred tank reactors showed that not even the presence of antibiotics could suppress the growth of undesired organisms. Sterile production technology became of paramount importance in mass cultures. The formation of pellets in submersion cultures posed another problem in that it prevented a sufficient supply of oxygen. Due to the lack of scientifically based biological process engineering, penicillin could only be produced in shake flasks. The specialists involved seemed to clearly underrate the problems they were faced with. Trial-and-error strategies were pursued without the contributions of engineers. It was only in later phases that the systematic development of efficient bioreactors for sterile production and high oxygen transfer was taken up in the USA and in England. Studies with sulfite suspension according to G. Tsao et al. [3] to assess the effects of vessel construction and mixing mechanisms were taken up and work on the scale-up towards large-scale production was undertaken. Thus, production of penicillin had increased to thousands of tons as early as 1948, despite the technical difficulties (Table 1).

The large demand allowed a rapid growth of the penicillin industry in the USA and, after the war, also in Europe. In the then German Federal Republic, Höchst in Frankfurt a. M. produced penicillin (see also [2]. In Switzerland Ciba in Basle, in close co-operation with the Institutes for Organic Chemistry and Special Botanics of ETHZ in Zurich, was very active in the research for anti-

Table 1. Annual production of the two first antibiotics (in kg)

Year	1947	1948	1949
Penicillin	24,856	57,513	80,076
Streptomycin	9,676	37,709	83,699

biotics with special emphasis on strain selection and development, as well as small-scale production for chemical and clinical purposes.

The increased availability of antibiotics reflects the important breakthrough in the industrial use of biology. It is the result of concurrent forces of various domains in the natural- (biology, chemistry) and the engineering sciences. The originally wild strain of *Penicillium notatum*, isolated by Alexander Fleming in 1929, only 10 years later yielded only 1.2 – 1.6 mg/l of nutrient medium. The increase in this yield remained a constant challenge to industrial research. Screening for potent wild strains and above all mutation and selection have led to impressive results in the course of the last decades (Table 2). Thus, an strain isolated from molasses, *Penicillium chrysogenum*, became the favourite of the penicillin industry. Mutants today yield over 30 g/l, which equals a 2000–3000-fold increase compared with the wild-type form.

Today, around 10,000 antibiotic substances are known and 1500 of these have been characterized. Around 90 substances are produced on a large scale. Of some there are known chemical derivatives with especially desired qualities, the screening for new antibiotics, however, has yielded fewer and fewer results and has been abandoned in many places. A very successful period of classical biology has thus reached the limits of its bioprocess strategies.

The antibiotics industry went through a phase of expansion in the 1950s and 1960s. A great number of new antibiotics were produced in large quantities, and the concomitant progress in process engineering was very impressive. As a result of medical progress, these products created a large added-value, despite their demanding production processes. Sterile submersion technology became

Table 2. Steps and efficiency in penicillin production. Scale-up step with batch mode. Continuous culture not efficient [5]

Typical process structure	
Petri dishes for maintenance of strains and (purity) testing	
200 ml shake flasks	agitated and aerated vessels; contamination-free downstream processing
10 l	
1000 1	
10,000 1	
100,000 1	
Process requirements for 100 kg penicillin G	
Electricity	300 kWh
Steam	4 t
Air	50,000 m^3
Cooling water	9,000 m^3
Process characteristics	
Modern processing	
Time for scale-up	7 days
Penicillin concentration	> 20 g per litre

Fleming in 1940 used shake flasks with 1.2 – 1.6 mg penicillin per litre.

the standard also for SCP (single cell protein) products from hydrocarbons or methanol, for ethanol from sugar cane (Brazil [4]), as well as for the production of vitamins and steroids.

Very early, bioethanol was used as fuel in Brazil. Hoechst, the German chemical company was brought into the process development by P. Präve, a successful industrial researcher, a prominent champion of modern biotechnology in Germany, and the first author of the standard textbook "Handbuch der Biotechnologie" [4a].

Technical microbiology of that time pioneered a development which led to the technology of the 1960s. Antibiotics became the market leaders among biological products. Once patents had expired and the cost for the treatment of the effluents and carriers had risen, the added-value of these production processes sank and they became bulk processes, which were, in part, relocated to Third World countries. Genetic engineering supplemented the mutation/selection strategy by targeted changes and it also allowed the synthesis of substances produced by the human body in microorganisms or cell cultures of human, animal or plant origin.

2
Genetic Engineering and High-Tech Mass Culture of Cells

Modern biotechnology is based on genetic engineering on the one hand and high-tech engineering for mass culture of microbes and higher cells from the living world on the other hand. In combination, the two have dramatically changed the scope of their use in medicine, agriculture and industry, and today even the environmental sciences have harnessed them to their tasks.

Their field of application has expanded beyond small scale and industrial fermentation, where – at least in the production of antibiotics, vitamins and enzyme-based substances – they are still unrivalled.

The impressive consequences of genetic engineering were particularly noticeable in agriculture and medicine, which – above all in the USA – led to the perception of genetic engineering as biotechnology per se. This attitude is less pronounced in Europe, since process engineering in chemistry – ever since Pasteur's microbiology – can look back on a long tradition and has made important contributions to industrial biotechnology. This latter is less disputed than genetic engineering, which for political reasons is facing major opposition in agriculture and the food industry and less critically viewed in medicine.

A quick look at the history of its evolution may prove useful for a factual appraisal and the comprehension of today's situation in the German-speaking regions of Europe.

2.1
Genetic Engineering

Genetic engineering is based on molecular biology, which itself was launched by research on bacteriophages and the knowledge of genes acquired from microbes in the 1940s. This was the starting point for genome research, which deals with

the structure and the function of DNA. In 1869, Miescher in Basle was the first scientist to isolate DNA from spawn and gave it the name "nuclein". At about the same time, Mendel was engaged in cross-breeding thousands of peas or beans and – based upon this research – formulated the three rules of hereditary transmission named after him. But neither he nor Miescher were able to link his findings to DNA. The first direct proof that genes – as functional subunits of the DNA-strand – were the hereditary transmitters was adduced in 1937 by M. Delbrück, Berlin, while engaged in research in the USA. In 1941, W. Beadle and E.L. Tatum were able to prove that in a filamentous fungus one gene was responsible for coding one enzyme. In addition to the gene transfer induced by bacteriophages (Delbrück and Luria 1943), conjugation by sexual reproduction of protozoa (J. Lederberg and E. L. Tatum 1946) and transformation by introducing DNA into a functioning cell (O. T. Avery, C. C. M. McLeod and M. McCarthy 1944) were identified.

Independently of these breakthroughs, the group around Monod at the Pasteur Institute in Paris detected conjugation in 1941 and, later, the linear organisation of genes in the genome of *E. coli* (1956). Working at the same institute, Jakob and Wollmann characterized the mechanism of genetic expression. The first step is the activation of a gene followed by the transformation of information by transcription (transforming the information from DNA to RNA) and translation (transformation to t-RNA). They described the whole process (operon) consisting of operator, repressor and structural genes. With the help of biological elements, one operon encodes on/off-functions similar to closed loops with control loops and electronic circuits. Biological control loop technology includes retroaction and can thus regulate synthesis and degradation of metabolic components.

The discoveries made in molecular genetics in defining genes and detecting gene expression and the research in gene chemistry were of equal importance, but it was the latter that boasted a breakthrough in 1953, when L. D. Watson and F. H. G. Crick, both in Cambridge/UK, identified the double helix. In 1970, Khorana, Madison/Wisconsin, performed the complete synthesis of a gene. This impressive result proved that four purine bases were sufficient to achieve the necessary specificity, if the pairs of bases A–T and G–C were lined up accordingly. Three years later, H. Boyer and S. Cohen were able to introduce the gene responsible for streptomycine resistance in a *Salmonella* strain into *E. coli*. This represented the first horizontal gene transfer with bacteria, and in 1976 it was again Khorana who was able to induce a foreign cell to express a biochemically/chemically synthesized suppressor t-RNA gene as it is found in *E. coli*. This established genetic engineering in the proper sense, and in a faster and faster rhythm – at first medically important substances – insulin, human growth hormones and human interferon were expressed by foreign genes in *E. coli*.

Since then, gene engineering has made great progress. Dozens of recombinant pharmaceuticals are on the market today and new products are being added all the time. Genetically engineered products more frequently replace enzymes in biochemical syntheses or in the food industry (rennin replacing rennet). One of the early products not for medical use was a bacterium used in cultures threatened by frost. The initial fears of its use in the natural environment were allayed by the favourable results of wide-ranging studies in the USA.

2.2 High-Tech Mass Cell Culture

The impulses of the pioneers (Novick and Szilard, Monod, Malek and others), who were keenly interested in the kinetics of biological processes still influence today's biological process engineering to a large extent. They developed new models for the mass culture of cells in continuous systems, which allowed them to calculate the kinetics of these processes quantitatively by either controlling the influx of nutrients (chemostat) or the maintenance of constant cell density (turbidostat). In this way, Monod developed his model, which describes the relation between substrate and cell mass. The chemostat method demands a high standard in experimental equipment, which in the 1940s was not reached. Critical points were sterility in mechanically agitated and aerated reaction vessels, air flow and the substrate supply from storage and collection vessels. Improvements in the control of processes by keeping growth factors such as temperature, pH and pressure, as well as oxygen supply constant were also indispensable. It was only in the 1950s that a few research teams in England, Sweden, Prague and Zurich began to take up this challenge. In 1958, the first symposium on continuous culture was organised in Prague and has become a regular bi-annual event in Western Europe. The chemostat method not only contributed to the development of process engineering, but also to the understanding of metabolic turnover in living cells.

In 1959, ETH Zurich [5] began establishing co-operation with the local industry which was engaged in developing and manufacturing new types of reaction vessels and in improving measuring and control technology. Within 10 years, numerous new developments were put on the market and chemostat technology turned out to be a high-tech technology for the bioindustry. Co-operation with ETH Zurich over many years gave several Swiss manufacturers a clear advantage on the global market.

Technological progress opened up new possibilities for research in metabolism and its regulation. Autonomous and dependent effectors were identified. Classic problems such as the Pasteur and Crabtree-effect, which had been the cause of clashes of opinion in yeast research for years, were elucidated. In addition to glucose, oxygen was also identified as an independent effector. Characteristics of various types of regulation of decisive metabolic processes were identified (A. Fiechter, G.F. Fuhrmann [6]; O. Käppeli and B. Sonnleitner [7], B. Sonnleitner and O. Käppeli [8]). This success was largely due to research on the composition of the media, which eventually led to transparent concepts for the design of media. Starting with yeasts and bacteria, these concepts were then successfully applied to cell cultures as well, and there made use of chemically defined media without serum addition possible (F. Messi [9]; C. Gandor [10].

In the 1960s, the efforts to synchronise the cell cycle showed that biologically regulated processes are extraordinarily finely tuned and precise. It was then still impossible to get beyond two or three synchronised generations of cells and the methods used in monitoring the maturation of individual cells by their enrichment with trehalose was highly complicated and demanding (M. Küenzi [11]).

Table 3. Innovative equipment developments for improved and safe chemostat experimentation (1959–1974 at ETHZ [5a])

Sterilization and scaling technology	High performance bioreactor	Measurement and control
Air filtration with ceramic filter, spring loaded, steam sterilizable	Internal loop flow COLOR type compact loop reactor (diameter: height = 1:1.1)	pH-Sensor shock proof sterilizable in situ
Peristaltic pumps for low delivery capacity and sterile operation	Mechanical foam destroyer on top	Combined glass-reference electrode
O-ring packings for piping and reactor parts	Short mixing time < 1 s for lab size scale	Hysteresis-free sterile operation housing
Membrane/needle closure for sterile air/liquid delivery	Homogenous gas hold-up	On-line system for effluent gas measurement O_2 (0–1%) CO_2 (0–3%)
Dynamic sealing instead of stuffing boxes		

Synchronisation of cell growth was shown to be dependent on the technical set-up of the growth experiments. It took more than twenty years to stabilise synchronisation in high yield chemostats (Münch et al. [12, 13]) and to reproduce changes in a cell population from the birth to the death of cells.

Chemostat technology has permanently influenced process engineering and the development of appliances as well as of plants and has thus prepared the ground for modern biological process technology. The crucial contribution to this success came from a new generation of chemostats, which permitted extremely precise control of the important growth factors and, consequently, modern process design. The system simultaneously worked with 40 signals online generated by control circuits and programme regulation [14].

The introduction of digital regulation replaced the former analog control circuits (temperature, pH) and widened the scope of process control and design. In addition to synchronisation, measuring of metabolic indices and of the involved substrates, intermediate and end products was introduced. The history of the advent of computers in biotechnology is the subject of a stimulating article by Harry Bungay in this issue [15].

Today, scientists have an extensive array of analytical methods at their disposal: photometry, HPLC, GC analysis and MS online. Samples of submersion cultures are made available with the aid of a tapping and preparation unit. Complete R & D programmes can be run automatically nowadays and automation will foreseeably take over production processes. Historically, automation has strong roots in Switzerland, as is shown by the contribution by W. Beyeler et al. [16] in this issue. With the current period of automation, a century comes to a close that – once it had overcome the myth of spontaneous creation – has tried to establish control over spontaneous natural processes by simple means.

2.3
The Post-War Period: New Products and the Emergence of Biotechnology

In the 1950s and 1960s, the development of production processes for antibiotics prepared the ground for modern biotechnology. The pharmaceutical industry recognized the advantages of biosynthesis and developed chemically produced derivatives; in addition it continued its screening for new antibiotic substances. New classes of substances were also introduced, such as the ergot alkaloid, cortisone, oral contraceptives and a number of other drugs. The industrial production of vitamins and of several nutritional amino acids had become possible. The latter field had been opened up by Japanese microbiologists (T. Beppu [17]; H. Kumagai [18]. As a consequence, for 20 years the Japanese industry was practically a monopolist for these substances until DEGUSSA, Germany, was able to become a competitor for a few amino acids, thanks to co-operation with H. Sahm and Ch. Wandrey, then on the staff of the Jülich Research Centre (KFA GmbH). The Japanese advantage over the competitors was due to the numerous microbiologists usually employed by the Japanese food and fermentation industry. They developed suitable strains for culture from wild strains they had carefully vetted and isolated in their laboratories. Some firms assumed a leading position not only in non-pharmaceutical products but in amino acids, polysaccharides and enzymes as well. Microbiology was given university status over 100 years ago; many of the post-war scientists were sons of sake brewers. The best-known names of this post-war generation were S. Fukui (1926 – 1998, Honorary Doctorate of ETH Zurich) and H. Yamada (* 1935, Corresponding Member of the Swiss Academy of Engineering Sciences SATW), who, to European scientists, represented Japanese biotechnology and were the first to bring Japanese biotechnology to Europe. In addition to microbiology, enzymology was highly developed and had a productive effect on single cell protein (SCP)-technology. Immobilising enzymes or whole microbes opened up new possibilities in biosynthesis.

At the time, large scale production of microbes aimed at producing protein for animal (and human) consumption on the basis of carbohydrates. Processes using Candida-yeasts as formerly used with wood sugar played an important role. Alkanes and various fractions of crude oil, later also methane and methanol served as substrates for bacterial SCP. The first research was undertaken in France (Champagnat [19]). Soon Japan, England and Germany joined the effort to develop production processes geared to 100,000 tons a year. Their process engineering was based on the latest findings in chemostat technology and on research on the regulation of the central metabolic processes in the degradation of alkanes as compared to glucose (A. Einsele et al.; A. Fiechter [20]).

The advantage of low cost substrates, however, was offset by the sizeable expenditure for processing. Under pressure from the farmers' lobby (under the pretext high cancer risks), ridiculously high demands on the purification of products were made, which put the limit for residual hydrocarbon far below that of common baker's yeast. Economic reasons were also responsible for the failure of the new branch of industry to develop. SCP triggered off a massive technological advance in the construction of reactor vessels and in process

design for biological processes and thus put the technology on the level of modern biotechnology.

In two papers for the annual meeting of DECHEMA in Frankfurt 1969 [21] and the 2nd Conference on Technical Microbiology in Berlin 1970 [22]), the German microbiologist H.-R. Rehm gave a comprehensive view of the influence of "modern microbiological-technical fermentation on the development of process engineering" and pointed out the advances in process design [22]. To him, the newly constructed reaction vessels for sterile processes, sterile measuring apparatus and sterile devices to combat foam formation and to supply oxygen with sterile air were of particular interest. He also took up growth and product formation in fermentation in three types according to the availability of carbon and energy sources for the formation of the main product according to Gaden. Further, he explained the state of the art in research on oxygen transport in heterologous systems of submersed cultures, which had been widely adopted for most microbiological processes. He also dealt with continuous cultures and the use of hypersensitive cells (cell cultures with more highly developed cells). Four years later, DECHEMA presented the programme for the promotion of biotechnology, which the Federal Ministry of Research and Technology (BMFT) was in charge of. H.-R. Rehm was responsible for the drafting of this programme [23].

With his roots in microbiology [23a], Rehm paved the way for modern biotechnology in Germany and as consultant was solicited by institutions of research promotion of the "Bund" (federal level) and of the "Länder" (state level), as well as by DECHEMA. On the occasion of the founding of the European Federation of Biotechnology, he was co-chairman and in 1981, he initiated the "Comprehensive Treatise of Biotechnology", today comprising 14 volumes.

The possibility of recombinant processes with bacteria is mentioned in this study, but not explicitly in the financial plan for specified programmes. Among the five research foci the very substantial promotion of sewage sludge and waste water disposal produced by the bioindustry are particularly noteworthy.

Apart from process engineering, this promotion was essentially aimed at research on "biological" and "special processes" and proposed a total of 242 research staff and roughly 570 million DM over five years. The implementation of this proposal by the BMFT started off biotechnology in Germany. Its ensuing rapid rise influenced many neighbouring countries, in particular the German-speaking ones.

The birth of this proposal, which was decided upon at the DECHEMA Conference in Tutzing (1972), is also noteworthy. It was compiled in only two years by 41 scientists from industry, universities and other research institutions and was the first document of its kind world-wide. Furthermore, DECHEMA created working parties, which examined the engineering of biological processes, their biochemistry and their biological bases. This study broke new ground and was up-dated several times. It constituted the basis for research promotion in biotechnology by the BMFT. 1978 saw the foundation – on Switzerland's initiative – of the European Federation of Biotechnology in Interlaken. With its secretariats in Frankfurt, Paris and London, EFB has contributed to the continuous development of the quickly expanding biotechnology. The

Conference on Biotechnology held at the same time in Interlaken convened 700 participants from 35 countries [24]. Courses of advanced training in biotechnology, also organized by ETHZ/DECHEMA, started at the ETH Zurich in 1973 and have become a regular feature in Switzerland and Germany, as well as in numerous other countries. They are of paramount importance in the training of young scientists. Membership in EFB has grown from initially 30 to over 80 societies in 24 countries today. EFB is widely recognized as representative of biotechnology in Europe, both on a scientific-technical level and in research policy.

In Germany, the initial efforts led to two large research institutions. On the initiative of M. Eigen and H. H. Inhoffen in Braunschweig, the existing Society for Microbiological Research – created by the Volkswagen Foundation – became the Society for Biotechnological Research (GBF) in 1974. It is active in all fields from screening to molecular genetics and from chemistry to process engineering, and as early as in the 1980s employed more than 600 staff.

The other large research institution for biotechnology is at Jülich. It is part of the former Institute for Nuclear Research (KFA), which used its restructuring, forced upon it for political reasons, among other things to integrate biotechnology. K. H. Beckurts, Director of Siemens (Berlin), later killed by the Red Army Faction, was largely responsible for this re-orientation. He implemented a concept that amalgamated the four existing institutes for Microbiology (H. Sahm), Process Engineering (Ch. Wandrey), Environment (C. J. Soeder) and Biochemistry, and established co-operation with Enzyme Technology (M.-R. Kula) at Heinrich-Heine-University in Düsseldorf.

In addition to these two centres of activity, by and by, a number of institutes at German universities have taken up different fields of biotechnology. At the time, a solid scientific and technological basis for "German Biotechnology" existed and a positive attitude prevailed. It was not a shortage of excellent scientists, a dearth of promotion funds by BMFT or the lacking willingness of the industry to co-operate, but political fundamentalism and ideological tenets that stopped promising developments, such as genetic engineering, and were also directed against the co-operation of the research-based industry with universities. Industry reacted by shifting research and production in certain fields abroad. Many promising young scientists followed this move. Well into the late 1980s, Germany lost precious time, which other countries, above all the USA and Japan, used to make an enormous advances.

2.4 Biotechnology in Switzerland

In Switzerland, as in other countries, it was in the first place molecular biology that paved the way for the development of modern biotechnology. In the wake of bacteriophages research and the chemical analysis of nucleic acids a re-orientation of biology took place. Starting with the simple microbes, the concept of the genes and their function as well as of complete parts of the genome were characterized. In this process, the scientists developed methods which they were able to use on highly-structured human, animal and plant cells, which soon changed the whole picture of biology. Apart from classical descriptive biology an ex-

planatory "new biology" emerged. Also, an important contribution came from protein research, which successfully unravelled the structure of the (complex) products of gene expression.

New biology was soon able to offer more and more rational explanations for the processes of life on the molecular level, to quantify and to model these processes. The conditions for their use in industry, medicine and plant cultivation were thus dramatically changed.

Biotechnology followed molecular biology. Whereas the latter produced two Swiss Nobel laureates (Arber 1978, Zinkernagel 1996), the former enjoyed a short period of expansion only and soon faced political obstacles in the wake of the events of 1968 in Germany. Low acceptance and very restrictive regulation led to subdued progress in R&D and in the transfer of results. The decisive development in genetic engineering took place in the USA, where to this day the largest number of recombinant pharmaceutical and agricultural, as well as nutritional products have been developed. Switzerland has lost its top position in a promising field as a new study by the chemical industry in Basle [25] seems to indicate.

In the 1960s, molecular biology was not ready for application in the form of genetic engineering. Restriction enzymes, able to cut the chains of nucleic acids at specific points, and performance vectors were still to come. In growing numbers, microbiologists joined the physicists and chemists in this new discipline. They were more interested in explanations for processes than in their description. In addition to microbes, the scientist used eukaryotes as objects of their research and thereby also put cell biology on a molecular level. The technology of mass culture was taken over from technical microbiology [26] and adapted to the use in cell and tissue culture. It became feasible to work with cells without walls in mechanically agitated submerse chains [27] and to replace complex additives such as fetal calf sera by chemically defined ones [28, 30].

In Switzerland, Werner Arber (Geneva University 1959 – 1970, and Basle University from 1971 onwards) was the first to isolate restriction enzymes and thus created the basis for what was later to be biotechnology. For this achievement, he was awarded the Nobel Prize in Medicine, together with D. Nathans and H.O. Smith. Identification and manipulation of genes later became a routine task and made the first lateral transfer of a foreign gene into *E. coli* by Cohen and Boyer possible. With the correct expression of the product of this gene genetic engineering had become reality.

For reasons of research policy, the U.S. Administration later equalled biotechnology with genetic engineering and by this did not simplify matters on either side of the Atlantic. In Europe, bioengineering and genetic engineering are subsumed under the more general term of biotechnology. The Swiss National Science Foundation (SNSF) uses the same definition for its National Research Programme "Biotechnology", since it aims at promoting process engineering, in addition to genetic engineering, through the use of computers in measuring and controlling processes, in on-line analytics and robotics for taking and analysing samples. This represents a quantum leap for process design and production control [29].

The concept of modern biotechnology as an amalgamation of genetic engineering and (bio) process engineering has become a tradition in Swiss

science policy and is based on the decisions by the Committee for the Promotion of Science and Research (KWF) – today Committee for the Promotion of Technology and Innovation (KTI) in the Federal Department of Economy. The committee was created by the Swiss Federal Government to foster the transfer of technology. 35 years ago, it was for the first time confronted with an application for a research grant in microbiological engineering from ETH Zurich. The application was supported by Aurelio Cerletti (a physician by training and a member of KWF) and the grant was awarded. As a direct consequence of this decision, Swiss manufacturers were in a position to penetrate 30 % of the global market in technical equipment for submerse culture.

2.4.1 *Biotechnology and ETH Zurich (ETHZ)*

The Federal Institute of Technology (ETH) in Zurich was the obvious site for the creation of a discipline combining biology and engineering. The conditions were favourable, since biology in the form of botany and zoology had been taught at ETHZ since its foundation in 1855. Later, disciplines of particular interest to agriculture (plant morphology, cattle breeding, microbiology, entomology) were added. Further disciplines for special training of natural scientists and agricultural engineers supplemented the offer at ETHZ.

In 1963, molecular biology made its first appearance, when R. Schweizer became a professor and was given the task to found an institute of molecular biology and biophysics by the then President of ETHZ, H. Pallmann. Schwyzer was one of P. Karrer's disciples – Nobel laureate of 1937 in chemistry – and had done some work on vitamins and antivitamins. The field of his choice, however, was peptide chemistry. He had developed methods for the cyclization of polypeptides and was the first to synthesize gramicidin, a dekapeptide with antibiotic effects on gram+ bacteria. Schwyzer considered his field as part of a new biology and defined molecular biology as structural biology in molecular dimensions. To him, the understanding of this dimension was part of its function. For this reason, biophysics were considered part of the institute's make-up, and K. Wüthrich was called in to build up a research group in nuclear magnetic resonance. This group was first housed in the institute of Richard Ernst (Nobel Laureate 1991 in chemistry), where it opened up new possibilities to protein research by developing methods to avoid crystallisation. Nuclear-Magnetic-Resonance(NMR)-spectroscopy made it possible to characterise large molecular structures, such as the BSE-proteins. In 1998, Wüthrich was awarded the Kyoto-Prize in recognition of his work in this field.

The successful development of this institute is a shining example of a farsighted political decision and was the first step towards restructuring biology at ETH Zurich. The far-seeing planning process of ETHZ allowed for the housing of the Institutes of Microbiology I (Ch. Weissmann) and II (M. Birnstiel) next to the newly founded Institute of Cell Biology with its four chairs. Under the guidance of ETHZ President H. Ursprung, the concept of botany/zoology was given up for the benefit of cell biology and specific institutes for plant and animal sciences.

In microbiology, first the chair for Technical Microbiology was created, transferred to its actual location on the Hönggerberg campus of ETHZ and developed into the Institute of Biotechnology (A. Fiechter) by 1982. To house biotechnology and microbiology in the same compound was a logical step.

In the meantime, the first recombinant pharmaceuticals had come on the market, e. g. human growth hormone, insulin and tPA – another substance produced by the human body and used to dissolve blood clots in blocked vessels.

Biotechnology, including its pilot plant, was installed at ETHZ at the right time. At the Institute of Molecular Biology at Zurich University, Ch. Weissmann had expressed Interferon α with a strain of *E. coli*. It was the first cytokine to be produced – in collaboration with the Institute of Biotechnology – in a modern 3000 litre bioreaction vessel in Switzerland.

The restructuring of biology and the development of biotechnology at ETHZ took place during the period of financial cuts in the 1970s. Co-ordination, the establishing of priorities and reallocation of budgets and personnel allowed the creation of new foci. Following the ideas of President Ursprung, the re-allocation took into account developments initiated by researchers such as J. Watson, F. Crick, F. F. Jacob, J. Monod and W. Arber. Ursprung considered the new biology and therefore biotechnology as well as "hard sciences" and predicted their important influence on chemistry, agricultural sciences, pharmacy and medicine. He was also convinced that biotechnology would one day have to pass the test of practical use in industry, and he came to the conclusion that in preparation for this step science policy had to be complemented by technology policy.

Looking back, his judgement was correct. The involvement of the Commission for the Promotion of Science and Research (KTI) in the 1960s marks the beginning of Swiss technology policy and also of biotechnology. KTI fosters application-oriented projects by grants of up to 50 % of the project budgets, if industry manifests its interest by putting up the other half. As has already been mentioned above, the inclusion of biotechnical engineering in this promotion programme had a positive influence on the manufacturing industry, small and medium-sized enterprises in particular. The origins of most of the manufacturers of biotechnological equipment can be traced back to this period. ETHZ, of course, was not the only institution to develop biotechnology. Similar endeavours were noticeable abroad.

Several stimuli encouraging co-operation between foreign groups came form the International Union of Pure and Applied Chemistry (IUPAC). At the suggestion of the Swiss representative, IUPAC began work on recommendations for university training in biological process engineering. Since chemical process engineering in Anglo-Saxon countries could look back on a long tradition, it was only natural that the first draft of the guidelines was influenced by leading experts from the USA: E. Gaden, R. Finn, M. Johnson, A. Humphrey, H. Bungay among others. Stimulated by the success of the antibiotics industry, they had started to study this new field and widely used computer technology to model growth and microbial production processes as well as the transfer of substances in submerse cultures. This spearhead of research interpreted biology as molecular, micro- and cell biology, enzymology and the study of metabolism. They gave their support to the endeavours of IUPAC and made extremely valuable

Table 4. Biotechnology research programmes at ETH Zurich 1959 – 1992 – in the fields of Biology and Genetics: Metabolic regulation, enzymes, membranes

Programme	Refs.
Growth of yeasts on glucose. Regulatory effect of oxygen and glucose	[31 – 38]
Enzymes, membranes	[39, 39a, 40]
Anaerobiosis	[41]
Hydrocarbon degradation	[42, 43]
Thermophilic bacteria	[44, 47]
P-450 studies	[48, 52]
Synchronization of cell cycles	[52, 53]
Lignin degradation research	
Enzymes involved	[54 – 58]
Models	[54 – 59a]

contributions to the discussion. In 1971, the draft for the training and the definition of biotechnology was submitted and adopted by the 1st IUPAC conference in Kyoto. Thus, an internationally accepted concept of this new discipline reached Zurich, where the restructuring of biology was in progress. The IUPAC recommendations had a major influence on the considerations concerning a technically oriented biology and positively affected the development of in-depth training in biotechnology. The Swiss Federal policy of research promotion helped to give a medium-term R & D strategy its shape. This strategy was directed at catching up first with the improvements of high yield processes and chemostat methodology and at widening the scope of reaction kinetics (Table 4). Secondly, it closely looked at the biological regulation mechanisms in the development of new processes which aimed at higher yields in general (Table 5).

2.4.2
Biotechnology in other Swiss regions

The Basle Biocentre. The Basle Biocentre was founded as a centre of competence in new biology, which pharmaceutical research of the Basle chemical industry thought indispensable.

The initiators of this centre were A. Pletscher (Hoffmann-La Roche) and E. Kellenberger (Geneva University). In the eyes of the Basle industry, the centre with its independent biological research should form the complement to their own (applied) research divisions. There was no call for biological process engineering, since the know-how of chemical process engineering was readily available in the companies.

In the early 1950s, Kellenberger founded the "Laboratoire de biophysique" of Geneva University, which was the precursor of the later "Département de biologie moléculaire". Kellenberger had devoted himself intensively to molecular biology and had recognised that only a new science policy could further traditional biology. He propagated his ideas in Switzerland and abroad in his lectures and by his research work. In 1961, he organised the first workshops and courses on phages. He was a co-founder of the European Molecular Society

Table 5. Biotechnology research programmes at ETH Zurich 1959–1992 – Engineering and process developments

Programme	Refs.
Measurement and control, automation, biosensors	[60–64]
Bioreactor design and testing	[65–71]
Human cell culture with melanoma and china hamster ovary (CHO) cells	[72–74]
Plants as raw material	
Proteinization of straw	[75]
Lignin degradation	[76]
Hemicelluloses	[77–78]
Acetyl-esterase	[79]
Pectinolic enzymes	[80]
Ramie fibres	[81]
Rhamnolipids	[82–87]
Thermophilic sludge treatment	[88–90]
Ethanol production with *Zymomonas*	[91]
Ethanol production with flocculent yeast	[94]
Methanol as substrate for bacteria	[93]
Methanol as substrate for yeast	[94]
Single cell protein (SCP) from molasses	[95]
Continuous production of vinegar with cell/liquid membrane separation	[96]
Stereoselective biotransformation with yeast	[97]
Lipoteichoic acid (LTA) an antimetastasic drug	[98]
Scaling-up of α-interferon	[1]

(EMBO), of which Switzerland became a member. In 1967, he initiated the Swiss Committee for Molecular Biology, which awards a certificate to successful participants of its courses. Finally, in 1979, Kellenberger became a co-founder of the Basle Biocentre, which became operational in 1970. From its very beginning, the centre combined microbiology and an institute of cell biology. Kellenberger also promoted electronic microscopy.

Since its foundation, the Biocentre has seen rapid development and has also integrated contributions in biotechnology from the neighbouring chemical companies, despite the centre's orientation in basic sciences. In addition, the biocentre is responsible for the training in microbiology in the framework of the "Ecole supérieure de biotechnologie de Strasbourg" (ESBS) to which the University of Freiburg i. Br. and TU Karlsruhe in Germany contribute plant sciences and bioprocess engineering respectively. This integration into ESBS made Basle University the first Swiss university after ETH Zurich to offer comprehensive training in biotechnology.

2.4.3
The Friedrich Miescher-Institute

In 1970, CIBA and GEIGY in Basle co-founded an institute for independent basic research in molecular biology. The foundation was named after Friedrich Miescher, who discovered nuclein. The institute's main activities lie in the fields

of molecular plant biology, cellular growth and neurobiology. In the context of Swiss biotechnology, the successful transfer of genetic information into monocotyledons, e.g. maize, was a notable feat of the institute. This success has since gained in significance, as it facilitated the development of genetically manipulated maize independently by two companies before the merger of Ciba-Geigy and Sandoz. From the point of view of research policy, it is important to note the transfer of work in genetic engineering of plants abroad, e.g. the USA. This situation is typical of many large European industrial groups, which carry out research in genetic engineering mainly in the USA

EPF Lausanne (EPFL). Among the universities in the French-speaking part of Switzerland only EPF Lausanne (EPFL) had the infrastructure necessary for the development of biotechnology. As the former EPUL, it had been part of the cantonal University of Lausanne until 1968, when it came under the management by the "Board of the Swiss Federal Institute of Technology" (BSIT) and changed its name to "Ecole polytechnique fédérale de Lausanne" (EPFL). It became the responsibility of BSIT to co-ordinate development of biotechnology within its writ.

For factual and financial reasons in view of the cuts in the budget, the BSIT decided in 1976 to base biotechnology at ETH Zurich to begin with. Since EPF Lausanne had no biology department and the department at Lausanne University was not geared to direct co-operation, the choice of ETH Zurich – with its technical microbiology and molecular genetics – was obvious.

EPF Lausanne was, however, determined to build up this new discipline as well. With this aim in mind, chemical process engineering (U. von Stockar) was expanded, and the Department of Environmental Natural Sciences created a chair of Biotechnology (P. Peringer).

In the course of a few years, the general restructuring of biology and the initiative of U. von Stockar as well as of J.P. Krähenbühl from the "Institut suisse de recherche sur le cancer" (ISREC) of Lausanne University made it possible to create a centre with five chairs. The "Centre de biotechnologie UNIL-EPFL (CBUE) houses bioprocess engineering (U. von Stockar), membrane research in the Department of Chemical Physics (H. Vogel), cell biotechnology (F. Wurm), molecular biotechnology (N. Mermod) and downstream processing (R. Freitag). Co-operation with other departments, e.g. "Sciences du vivant", working on relevant fields of biotechnology (biomaterials, biophysics), was established. As a result of the flexibility of a youthful EPFL, new forms of organisation in an expanding discipline had become possible.

Swiss Institute of Bioinformatics (IFB). The youngest institution in Swiss Biotechnology is the Swiss Institute for Bioinformatics (IFB), founded in 1997 in the French-speaking part of Switzerland. It was created by merging several groups in protein research at the universities of Geneva and Lausanne including ISREC (Swiss Institute for Cancer research). ISREC is primarily engaged in protein research at molecular level (proteomics).

3
Biotechnology in Medicine, Agriculture and Environment

In the last few years, genetic engineering, above all in the USA has made important progress and has triggered off new developments in medicine, agriculture and environmental sciences.

Interest became rather focused on the biological production process of human endogenous substances for therapeutic uses; substances that could not be produced chemically.

Research teams at US universities and their alumni proved to be particularly successful in this. With little capital expenditure they started small businesses, the so-called gene technology companies. The large chemical and pharmaceutical companies were rather reluctant to follow this development, since, at the time, registration of recombinant products by FDA was a very time consuming process. For political reasons, the chemical industry in Europe was even more reticent to invest in US gene technology companies. When they did, these companies were generally given a lot of freedom to define their own strategies.

On the whole, some 1500 such small companies were started in the US, not all of which, however, survived. The time necessary for development and the inherent insecurities in the registration process required a substantial financial basis, which not all of them had. According to a recent estimate, R & D cost of genetically engineered pharmaceuticals are roughly on a par to those of chemically produced drugs (around Swiss Fr. 500 million).

Table 6. Profiles of biotechnology development in USA and Europe in 1996

	Europe	USA
No. of Biotech companies	716	1,287
Total No. of employees	27,500	118,000
Average No. of employees per company	39	92
Company profiles		
Founded since 1986	45	36
% with > 50 employees	81	75
Revenues total Mio $	2,151	11,680
Average revenue Mio $	3.0	9.1
Losses, Mio $		
Total	11,113	3,750
Average	1.5	2.9
R & D spending, Mio $		
Total	1985	2320
Average	2.6	1.8
Genetically modified plants		
Registered	4	28[a]
Released	750	2500
Crop area, acres	0	1,481,000
Venture capital invested		
lst 6 months 1990, Mio Euros	80	533

[a] USA, Australia, Japan (excluding EU) [99].

The appearance of biotechnology in Europe was clearly delayed. Table 6 shows the advance of the USA in the number of new start-ups as compared to Europe. For the last few years, this number has decreased in the USA, but the large number of jobs (namely 118,000) created remains impressive.

Europe particularly chafed under the prevailing unfavourable conditions, when, in 1983, the first gene transfer in higher plants was achieved in the USA. In Switzerland, particularly restrictive regulations for the release of genetically engineered plants were passed, but even these could not prevent the willful destruction of fields for duly authorised plant trials.

As soon as genetic engineering was gaining ground, the thrust in the development of new biological production processes and new bulk products changed away from examples such as single cell protein from hydrocarbons or ethanol from cellulose as a replacement for fossil fuels. The only niche product remaining on the US and Brazilian markets is bioethanol from maize or sugar cane, purportedly produced without public subsidies. However, they do not represent a valid alternative to common fuels.

3.1 Medicine

With the biosynthesis of two human endogenous substances, genetic engineering achieved its first success. *E. coli* served as the host cell; for safety reasons an apathogenic strain had been chosen. This was the reason why its yield was only half that of analogous wild strains. However, it came fully up to safety expectations. The missing capacity of glycolysation, which only eukaryotes have, was another disadvantage. This is one of the reasons for the spreading use of yeast and human cells. The latter are particularly adapted for generating the desired glycolysation patterns.

Nowadays, pharmaceuticals are genetically produced on a scale that clearly surpasses the estimates of the 1980swith 40 products in 2000 and a turnover of Swiss Fr. 60 billion. In 1995 already, turnover was Swiss Fr. 40 billion, offering 300–400,000 jobs. The initial list of recombinant products (insulin, human growth hormone, tPA, α-Interferon, erythropoietin) soon grew longer. The first to follow were monoclonal antibodies for diagnostic and later for therapeutic uses (Table 7). In 1995, there were 120 products in the registration pipeline; this number had grown to over 300 in 1998. The ten top selling products generated more than $ 7.5 billion (Table 8).

The current programmes of human genome research are of the greatest significance to the future of biotechnology. This research encompasses the complete sequencing of the human genome, identifying the genes and characterising their interaction. The US Human Genome Organization (HUGO) is the leading institution. Research teams from Great Britain and France, the National Genome Research Institute (NGRI), as well as companies such as Celera/Genomics (Craig Venter and Perkin Elmer) are integrated into this research. In 1998, Craig Venter predicted the conclusion of this programme within 3 years, the NGRI expects research to take until 2003. At the same time, Incyte Genetics was even more daring to predict the sequencing of all genes,

Table 7. Monoclonal antibodies (MAB) for human therapy [100]

Product	Indication	Developer
ReoPro	High risk	Angioplasty Centocor
Rituxan	Non Hodgkin's lymphoma	IDEC/Genentech
Zenapax	Transplant rejection	Protein design labs
Remicade	Crohn's disease	Centocor
Simulect	Transplant rejection	Novartis
Synagis	RSV infection	MedImmune
Herceptin	Breast cancer	Genentech
CMA676	Acute myeloid leukaemia	Celltech
Beexxar	Non-Hodgkin's lymphoma	Coulter pharmaceutical
Oncolym	Non-Hodgkin's lymphoma	Alpha therapeutic
Anti-IgE	Asthma	Genentech
Panorex	Colorectal cancer	Centocor
IDEC CE9.l	Rheumatoid arthritis	IDEC

Table 8. Top Ten Selling Biotech Drugs 1997, 1996 [101]

Drug	Developer	Marketer	Indication Sales in Mio $	1997	1996
1 Procrit	Amgen	Ortho Biotech	Red blood cell enhancement	1169	995
2 Epogen	Amgen	Amgen	Red blood cell enhancement	1161	1150
3 Neupogen	Amgen	Amgen	Neutropenia reduction	1056	1017
4 Epivir	BioChem Pharma/Glaxo Welcome	Glaxo Wellcome	HIV	973	306
5 Humulin	Genentech	Eli Lilly	Diabetes	936	884
6 Intron	Biogen	Schering-Plough	Cancer and viral infections	598	524
7 Engerix B	Genentech	SmithKlein Beecham	Hepatitis B Vaccination	584	568
8 Betasaron	Chiron/ Berlex	Berlex/ Schering AG	Multiple Sclerosis	387	353
9 Genotropin	Genentech	Pharmacia & Upjohn	Growth failure	349	391
10 Cerdese/ Cerezyme	Genzyme	Genzyme	Gaucher's disease	333	265
Totals				7546	6453

including nucleotide polymorphism (SMP) for each gene, to be completed within one year.

The human genome is made up of around 3 billion sequences and 100 – 150,000 genes. They represent the smallest part of the human genome (perhaps 5%), the remaining DNA is formed by introns of still unknown function.

Genomics, the term created for genome research, combines automatic performance sequencing with a new domain of computer sciences, bioinformatics, for the processing of the enormous quantities of data. Sequencing is based on the amplification of small and smallest parts of defined DNA. This is achieved with polymerase chain-reaction (PCR), which was introduced in the eighties.

PCR has proved invaluable in forensic analysis to genetically fingerprint individuals without error. It is also used to diagnose hereditary diseases and is the basis of pharmagenomics, the emerging new discipline of individually adapted drug therapy. Human genome research will eventually help to find therapies against cancer, rheumatism, arthritis, osteoporosis, cystic fibrosis, multiple sclerosis and neural diseases, and will boost the endeavours to control angiogenesis in cancer therapy and to develop an AIDS vaccine [104].

Genomics on its own cannot register genetic processes. It has to be seconded by performance separation of genetically expressed proteins, their identification and their quantification. Today's technology allows registration and processing of 10,000 spots with 2D-PAGE-gel. Here again, we are faced with enormous quantities of data that can only be processed by new software. In analogy to genomics, this technology is called proteomics. A number of firms are currently engaged in linking genomics and proteomics to characterise interaction between proteins and their coding genes. The quantitative contribution of single proteins can be assessed only by this interaction, and thus show the hierarchic order of the complex system [107]. The announcement by Incyte Pharmaceuticals of a human genome clip (for $100) for the year 2001, once sequencing of human genome has been completed, show the impressive potential of the genome programme and gene firms.

It is foreseeable that this newly emerging industry will push genome research to its limits and help to gain new insights into the diversity of processes in living cells. Molecular processes at the lowest level will be characterized and attributed to the various types of cells. This again opens up new vistas on the next level of organisation, e.g. in organs and in complete cells. New technologies will be needed for this, which will probably be named cellomics [105].

In view of these perspectives, important progress in medicine in the next few years can be expected, which will allow for efficient patient-centred health care [106]. This will include the fight against pathogens that are partially or wholly resistant to today's therapies, such as HIV and hepatitis viruses, *Legionella*, *Borrelia*, *Heliobacter*, *Bartonella*, *Chlamydia*, against which new strategies have to be developed. Pressure on our immune system by infectious and parasitic diseases is not diminishing (about 16 million deaths every year), and preventive measures have to be complemented by specific antigens, vaccines, cytokines and others based on the genome, that make efficacious therapies possible. In view of the rising life expectancy and the limited financial possibilities of Developing Countries, strategies will have to be found to overcome the economic limitations

of therapies. Social and political measures will have to be accompanied by the development and production of new drugs, e. g. orphan drugs. An efficient solution to these problems is not thinkable without the giant programmes of genome research.

Genome research is also essential to biological process engineering. The production processes can be improved by a large factor by genetically modified biocatalysts (complete cells or their functional parts). Since mass culture in chemically defined (and economically viable) media has become possible, submerse cultures of mammalian cells are coming more and more into their own for the biosynthesis of endogenous therapeutics and their derivatives. Genetic manipulation can increase production, as an example from Switzerland shows. The construction of a multicistronic system of gene expression in CHO-cells achieved a 30-fold increase [108]. Fussenegger and Bailey at ETHZ managed to arrest the cell cycle in the G_1-phase by introducing genes that expressed a model protein SEAP, the cell cycle inhibitor p27 and the protein Bel-A_2. In this "resting" state, the whole carbon and energy contents of the cell can be used for the synthesis of the desired product. In addition to the multicistronic expression vectors, the introduction of a survival factor to prevent apoptosis is crucial to success.

The above procedure would have been impossible without the knowledge gained in the research of large genomes. The lagging behind is sorely felt in Europe, and viruses, bacteria and baker's yeast are no replacement as the HGP-programme shows. In 1989, it was still a maxim of EU-funded research that only ethically unequivocal subjects were accepted for genome research. This is the reason why *Saccharomyces cerevisiae*, a eukaryote with less than 14 million sequences and 6000 genes, was chosen. Towards the end of the research programme close on 100 laboratories were involved, they worked as a network and achieved their goal in seven years [109]. The only Swiss participant was the group of P. Philippsen at the Basle Biocentre.

In 1991, a group of experts activated human genome research in Germany. In the wake of a workshop on "Technology Development in Genome Research", they recommended a concept to the Federal Ministry of Research and Technology (BMBT) for the promotion of genome research over nine years and with funding of DM 35 million in the first three years. This suggestion resulted in the joint announcement by the BMBT and the German Research Society (DFG) of the start of the current German Human Genome Project (DHGP) in 1995. It has as its aim the systematic identification and the structural characterisation of human genes of particular relevance to medicine. By 1997, the programme had united nine central research institutions and 24 independent working parties, financed by the BMBT, together with two resource groups. Essentially, they are located in the Munich area (12 groups), in Heidelberg (ten groups and one resource centre) and in Berlin (eight groups and one resource centre). In addition, there are a number of independent groups in various places. 23% of the funds are spent on related sub-programmes, such as model organisms (mouse, rat, Drosophila, zebra fish), ethics, evolution and bio-informatics.

In addition to the DHGP, BMBT launched a programme for the promotion of biotechnology in nine geographical regions, in which universities and public

research institutions have established co-operation with industry. Within two years, the flexible management of this programme had brought together 234 partners by 1998, had reaped economic success and created a genuinely positive atmosphere [110]. In 1998, the DFG (under President E.-L. Winnacker) reviewed the DHGP and added a programme for bio-informatics. Within 5 years and with DM 50 million, two or three structured projects were to catch up with bio-informatics world-wide. For this to be successful, restructuring of universities and extramural research institutions has to be initiated to combat the lack of experts in bio-informatics. They are sought after by industry and outside Germany. The establishment of centres for bio-informatics abroad, such as the Swiss Institute of Bio-Informatics in Lausanne, has given urgency to the measures of the DFG. Towards the end of 1998, E.-L. Winnacker suggested a meeting to discuss the future of genome research. This resulted in the position paper of 1999, largely influenced by P. Bork (EMBL, Heidelberg, and Max-Delbrück-Centre, Berlin), which suggested the funding of research in bio-informatics with DM 1 billion for 5 years [111].

3.2 Agriculture

In 1999, I. Potrykus, Professor of Plant Science at ETH Zurich retired. Following the restructuring process in biology at ETHZ in the 1980s, he was the first to use genetic engineering for useful transgenic plants with a view to securing the basis of nutrition for people in underprivileged countries. In 1984, when he was still at the Friedrich Miescher-Institute in Basle, he managed to introduce a foreign gene into tobacco plants with the help of *Agrobacterium* as a vector. This method, however, was not transferable to all varieties of plants. This proved that the specific place the foreign gene takes up cannot be predefined and that gene transfer, at the time, was a difficult problem.

A few years later, then at ETHZ, Potrykus was able to directly integrate foreign genes – with the help of polyethylene – into the DNA of protoplasts, that is to say preparations of single cells without cell walls.

The protoplast method allowed the development of plants that were resistant to diseases and pests. His last breakthrough was a rice plant that is rich in provitamin A, which could be of paramount importance to 130 to 400 million people depending on rice as their staple food. The new variety has been handed over to the International Rice Research Institute in the Philippines to be crossed with varieties that are adapted to local conditions, so that the high provitamin A contents could be passed on. The improvement of cassava, an important food source in Africa, was another success of his and his Zurich team.

To cope with the rise in the world's population to 7.7 billion people in the next twenty years, food crop yields in agriculture will have to be increased considerably [112]. The rising population of Third World countries alone will necessitate an increase of food production by 80%. The increase in grain production will have to reach 40%, that of meat 63%, and 40% more roots and tubers will be needed. To meet this growing demand, there is 5.5% of uncultivated land available. The harvests by hectare will have to reach the usual average of 4 tons. This

Table 9. Research programmes in biotechnology at ETH Zürich 1992 onwards [102]

Metabolic engineering (Chair: James E. Bailey)			Biological transformation (Chair: Bernard Witholt)
Enhancement of particular pathways	Biochemical synthesis by *E. coli*	Basics	Architecture of alkane monooxygenase
	Bacterial selection of heterologous pathways		alk recombinants as hosts for other (eukaryotic) membrane proteins
Improved industrial micro-organisms	For controlled proliferation		Genetics, enzymology and regulation of biopolyestersynthesis and PHA synthesis in plants
	For high growth and bioconversion efficiency	Process developments	Growth of rec-*E. coli* and rec-*Pseudomonas* in 2-liquid phase media (10 – 50 %, alkanes and aromatic solvents)
	For oxygen-limited activity		High cell densities and stable long term oxidation activity for *P. oleovorans* and *E. coli*
Generation of New Molecules	Glycoproteins		Downstream processing in series with continuous cultures
	Secondary metabolites		Scaling-up and safety (explosion danger) in two-liquid phase bioreactors
New R & D Technology	Screening Technology		Integrated bioconversion-bioprocessing systems to convert substrate specific: products
	Monitoring of protein pattern	Products	Synthesis of aliphatic andaromatic alcohols, aldehydes, epoxides and acids by oxidation of aliphatic and aromatic compounds, with NAD(P), FAD, PQQ requiring mono-oxygenases, Axidases and dehydrogenases
	Mathematical modelling and analysis of metabolic systems		Production of poly-hydroxyalkaoates; (PHAs) by Ps. oleovorans alkanes Application of PHAs

can only be achieved by improved methods in agriculture. The use of transgenic crops in agriculture offers a solution. Agricultural crops even today can be adapted in many ways: resistance to insects, viruses, bacteria and fungus growth; enrichment in β-carotene to increase the provitamin A contents and the nutritional value (oils, starch, essential amino acids) ; specific profile in fatty acids; slowing down the maturation process, e. g. to prevent rot in melons and to solve storage problems; resistance to drought; tolerance against salt, aluminum or manganese in sour soil; improvement of cattle feed (higher cellulase or phosphorlipase contents for easier digestion, enzymes for toxin degradation); formation of antigens as oral vaccines. It is important to integrate agriculture in the Third World in this drive, since current marketing methods in industrialized countries are of no use there.

Among the first examples of such crops we find the new transgenic potato variety "Alpha", which is virus resistant. It is an item in the honest broker system of the International Service for the Acquisition of Agri-Biotechnological Application (ISAAA) in Mexico. The seeds are handed out to Mexican farmers, together with instructions for the planting and for the application of risk and biosafety methods according to CINVESTAV (a joint research institute of Mexico and Monsanto in St. Louis). In this way, the threat of monopoly by the big firms in the life science sector has been alleviated [112]. Such solutions take up I. Potrykus' idea of science based on ethics. Enhancement of farmers' chances in the developing countries has been the impetus for his research into crop improvement by genetic engineering. The successful transformation of plastids by him and his team was a real breakthrough which has cleared the path for new solutions to future nutritional problems.

Resistance to the use of genetic engineering in agriculturally used plants has taken unexpected dimensions. The early objections to genetic engineering itself and its use in medicine and the biosynthesis of pharmaceuticals in the 1980s eventually resulted in legislative measures, first in the USA, a little later also in Europe. This marked the beginning of specific safety research. The remarkably pragmatic approach of US legislation did not encumber carefully planned research. The Parliament in Germany took a different stance: in 1989, it passed such restrictive legislation on genetic engineering that it had to slacken the reins again in 1992. The accumulated data on plant trials show that German contribution to research and development is very modest and negligible compared with US achievements in this field (Tables 10 and 11).

Thanks to flexible regulation in the USA, not only have there been no accidents due to negligence so far, on the contrary, valuable know-how and insight into a large number of agricultural crops and garden plants has been gained.

This contrasts with events in Switzerland, where activists prevented the authorised release in two instances, and where an administrative decision has made the culture of genetically engineered maize illegal, although the Swiss voters rejected an initiative which aimed at completely banning genetic engineering in Switzerland.

Despite the unfavourable conditions in Switzerland, we can safely assume that in the wake of globalisation and the ensuing changes in agriculture, urgent problems will be solved with the help of genetically engineered plants. In the

Table 10. GMO Releases in the EU(cumulative data) [103]

Country	No. of Proposals
France	398
Italy	223
UK	174
Spain	124
Netherlands	108
Belgium	97
Germany	96
Sweden	36
Denmark	34
Finland	13
Greece	12
Portugal	10
Ireland	4
Austria	4
Total	1333

Table 11. Released GMO crops in the EU (cumulative data) [103]

Organism	No. of proposals
Maize/Corn/Sweet corn	359
Rape	279
Sugar Beet	210
Potato	147
Tomato	71
Tobacco	48
Chicory	37
Bacteria	34
Cotton	16
Soybean/Soy	12
Wheat	11
Poplar	10
Sunflower	70
Melon	10
Marigold	9
Others (34 additional organisms)	96

case of agricultural crops, the replacement of pesticides, qualitative improvements in view of a more balanced nutrition and the replacement of nitrogenous fertilisers by the synthesis of organic nitrogen produced by rhizobia will be the first step only. Though the synthesis of rhizobia has not been characterized yet [113], it remains a fascinating goal which will be reached in the medium term.

The newly emerging Health Partnerships formed by chain stores open up new aspects against the negative developments in the dietary habits of the affluent society in industrialised countries. These chains educate their customers and

offer controlled diets which will eventually replace chemical drugs. Within the next twenty years, food processing and distribution will make up for the largest share (84%) of the added value in the food industry [114]. The rest (7%) will come from farming and genetically engineered seeds (9%) produced by life science companies. It is estimated that the added value of this new branch of industry will be $ 15.36 billion. J. Calder summarises this development the following way [115]:"In the next few years, we will expect to see the continued development of crops with improved pest resistant traits, environmental adaptability, and products altered for desirable output qualities."

3.3 Environment

In the 1970s, the development of molecular biology and its application were extended to include environmental protection. The increasing strain on the environment caused by traffic and industry and the spreading urbanisation have mobilised scientific and political circles to protect nature. They first took an interest in the protection of the variety of species and the problem of the rising output of CO_2 by a rapidly growing consumer society. The use of biological technologies in integrated environmental protection was first directed at keeping the environment clean and at producing energy from biomass. This was the origin of a new technology for the production of energy and proteins from lignified parts of plants developed by ETH Zurich [54–59, 59a, 75–77].

Biotechnology in environmental protection mainly means biological process engineering for the treatment of effluents, mud, composting of garden plants and the cleansing the soil of toxins. Genetic engineering still is of little importance to this, it will, however, come into its own in the replacement of recalcitrant products from industrial processes (Table 12). It may safely be said that in future a large number of new enzymes will be available, produced by efficacious genetically engineered hosts. We will expect new genetically produced biodegradable substances such as surfactants, which for economic reasons cannot replace chemically produced substances yet [82–87, 119]. Next on the list after surfactants are sulphur and chloride [116] in the paper industry. The bleaching of fibres in paper production has seen remarkable progress. Nowadays, a complex enzymatic process is used, in the development of which the Institute of Biotechnology at ETH Zurich was involved in the 1980s. The first step consisted of isolating new enzymes involved in the degradation process (Table 5). The bleaching effect, however, was only partially achieved, and it could be proved that intermediate products negatively influenced the primary process [59, 59a, 117]. In the last few years, in-depth research has eliminated this undesirable retroactivity, and today, the bleaching process seems to be ready for transfer to practical application (K. Messner [118]). The long and complicated history of chloride replacement gives a good impression of the enzymatic and technical difficulties in replacing classic but environmentally problematic processes. The enzymes that can be used in the bleaching process are not produced by genetically engineered fungi. For economic reasons, we expect the development of genetically engineered high yield strains that are optimally adapted to the bleaching process.

Table 12. Selected enzymes contributing to sustainable development [105]

Industry segment	Enzymes	Chemical(s) replaced	Process(es) replaced
Detergents	Lipases, proteases cellulases, amylases	Phosphates, silicates surfactants	High temperature, energy
Textile	Amylases, cellulases, catalases	Acids, alkali, oxidizing agents, reducing agents	Energy, reduced machine wear
Starch (i. e. high fructose, corn syrup, fuel ethanol, etc.)	Amylases, pullulanases, glucose isomerases	Acids	High temperatures
Leather	Proteases, lipases	Sulfides, surfactants	High temperatures
Feed	Xylanases, proteases, phytases, cellulases	Phosphorus	Lower environmental phosphate and waste (manure) levels
Film silver recovery	Proteases		Recovery of silver from used film

High performance biological process engineering is indispensable for the treatment of effluent and solid wastes from industry, households and farming.

In addition to recycling (e. g. composting) or waste clearance (e. g. contaminated soils), particular importance is attached to effluent treatment, because of its high cost to the taxpayer. Waste water treatment produces large quantities of solid residue that are burnt or biologically treated. There are a number of established processes, subject to debate according to local conditions. Since aerobic processes produce CO_2 and H_2O, anaerobic processes, however, CH_4 and H_2O, nowadays, the latter are preferred to produce energy. Thermophile aerobic installations, however, offer excellent sanitation at temperatures above 50 °C ($< 10^3$ bacteria ml^{-1}) and, thanks to their simple layout, the processes can be easily controlled. [44 – 46, 120].

Thermophile aerobic processes offer the further advantage of being easily adaptable to local conditions in view of quantities to be treated and in optimizing the scope of effluent systems.

4 Political Aspects and Acceptance of Biotechnology

The first artificial gene transfer gave research a boost that, after a dozen years, led to the production of the first drugs with *E. coli* bacteria. Misgivings of the possible dangers for the environment and for human beings, as well as misuse in medicine soon cast a shadow over the positive prospects for the improvement of medical therapies and for the creation of a new branch of industry based on biological processes. In the USA, J. Rifkin gave up his university career to found an organisation which had as its sole aim the introduction of very strict political

supervision of this new technology. As it turned out, the Americans with their penchant for pragmatism showed a positive attitude towards the innovation genetic engineering brought about. In the USA and Canada, several opinion polls in the nineties showed a remarkable constancy in the perception and acceptance [115]. More than 70% of the population were positive towards research, genetically engineered food and crops. On the contrary, it thought biotechnology less risky than microbial contamination, pesticides or food additives, but drew the line at the cloning of animals. Tastier food, convenience and low prices were important to them. How seed was developed was irrelevant. Decisive for the acceptance were the long term information efforts of government, industry and universities, not to forget third-party groups, long before the first biotechnological products had found their way to the market.

The evolution could not have been more different in Europe, where government right from the start took a negative stance. The political left pointed out questionable procedures and unfathomable risks and thus prevented a wide and sustained acceptance. As early as 1982, FAST (Forecasting and Assessment in Science and Technology), a so called "future group" of the EC (European Commission) underlined the crucial importance of perception for acceptance or refusal of new technologies. Up to 1992, consumer and citizens organisations ran 10 workshops, which led to a number of publications intended for the general public. Their success with the general public was mitigated, they influenced, however, research, which tended to turn to "less risky" fields. Information and recommendations [121] of political organisations (7%), trade unions (6%), industry (7%), religious bodies (12%) met with little confidence, even public authorities (17%) were unable to convince the public, which believed environmental and consumer organisations (56%). The implied dangers were minimised by ever stricter legislation. In 1986, Denmark was the first country to pass a Gene Technology Act, followed by Germany with exceedingly restrictive legislation, which came into force in 1990. Political groups, such as "the Greens" were strictly against genetic engineering and its products. It was, however, not clear, whether this attitude was dictated by political opportunism or genuine conviction. The directives of the EC dealing with specific technological aspects of biotechnology were also disastrous for certain areas of research. Human genome research could only be continued in France and in Great Britain. Since 1995, Germany has tried to catch up with international research at mounting costs. In 1992, legislation in Germany had to be changed so as to allow for the start of a human genome programme, in which three centres and several outside groups are involved, and which is carried out under the auspices of the Federal Ministry of Research and Technology (BMBT). Although progress is promising, the German Forschungsgemeinschaft (DFG) thought it appropriate to add DM 1 billion over five years to the annual budget of DM 70 million of BMBT funds to complement and speed up the programme. These funds are intended to equal the $330 million over three years poured into biotechnology research in France and Great Britain. The DFG under its President E.-L. Winnacker has further launched a proposal for a "National Genome Initiative" directed by BMBT, and has called for the appointment of a "National Committee for the Co-ordination of Genome Research". In Germany, bio-informatics in particular have been left

at the post, and an annual sum of DM 100 million is thought necessary to catch up with research internationally.

The dramatic change in German policy also manifests itself in the growth of the bio-industrial sector of industry, which with 222 of 1200 small and medium-sized (SME) companies in Europe takes second place behind Great Britain. This figure rose by 28% in 1999 compared to 1998 and with 17% exceeds the European average.

The political development of the 1980s in Switzerland, too, had negative consequences, which took the form of specific legislation for this sector of research. It all began, as in the EU, with specific technical directives issued by the Safety Committee (SKBS) formed by the Swiss Academies. They started with guidelines to regulate non-human microbiological research. In the wake of manifold political activities and initiatives, a constitutional clause against improper use of biotechnology in non-human research areas was passed. It mentions respect and dignity of living beings, safety for humans and animals, protection of the variety of animal and vegetable species. The USA and the EU served as examples for the Swiss Government for a series of legislative measures specifically aimed at biotechnology, which will probably take until the end of 2000 to be passed.

In 1992, the Swiss political Left started an initiative "For the Protection of Life and Environment from Genetic Engineering (Gene Protection Initiative)", which wanted to add a clause to the Swiss Constitution and would have severely limited research in this field. In 1998, it was thrown out by a clear majority of 68% of 41% of the Swiss who took part in the referendum. The most remarkable event of the campaign was a demonstration – the first of its kind – of more than 3000 scientists in the streets of Zurich, as part of a campaign to fight the initiative and its disastrous demands.

Despite the clear defeat in the referendum, opposition against genetic engineering, especially against the use of genetically engineered agricultural crops is kept up by pressure on legislative bodies and the public administration for more restrictive measures. As a result, genetically engineered varieties of maize have been given clearance for consumption, but their controlled release was not authorised. Foods with a content of more than 1% of genetically engineered organisms have to carry a label. Genetically engineered organisms that have not been cleared for consumption are not allowed in foods at all (zero tolerance).

5 Outlook

We have shown that in the early phases of molecular biology Europe made essential contributions despite World War II. Basic research in the USA made such rapid progress that practical application of its results was soon envisaged. It cannot be denied that genetic engineering in the USA was very much more performance oriented, as the many new enterprises founded by microbiologists straight from university show. The USA are also the dominant force in genome research, the characterisation of complete genomes and the interactions between single genes, as well as of their products. New disciplines emerged such

as genomics and proteomics, which produce enormous quantities of data that call for new approaches in data processing. Bio-informatics is the key to the interaction of genes and its regulation and will make characterisation of processes on the lowest molecular level possible.

This clears the path to molecular structure and the organisation of whole cells and the "common buzzword" for the molecular biology of organelles and whole cells will be cellomics.

Cellomics will be the basis for a number of applications in medicine and in a wide-ranging bio-industry, which will come up with new therapies based on new human endogenous substances and new strategies for cell and tissue-cultures. It will give insight into still incurable genetically linked illnesses and into realistic genetic engineering. Opposition against the use of transgenic crops will diminish in favour of a more realistic judgement.

The first signs of this development in German-speaking Europe are discernible in Germany, where the Federal Minister of Research not only started a human genome research programme (1995), but with the "Bio-Regional-Programme" (1997) also stimulated industry to create a performance bio-industry The new coalition government formed by the Socialists and the Greens have confirmed the preceding government's policy of "Biotech Business as Usual" and intends to keep up research promotion as well as possible, despite cuts in the Federal budget.

This increases pressure on Switzerland not to follow others in genomics, but to take a lead in relevant areas. It can start from a comfortable position in proteomics, bio-informatics and the biotechnology of plants. Its international ranking is excellent, and, if it were encouraged by a prospective and courageous research policy, Switzerland could make manifold valuable contributions to the shaping of the emerging landscape in the biology of the new century.

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